

**Mechanism of Growth Inhibition by cAMP
Analogues on Cultured Cancer Cells**

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DECLARATION

In accordance with regulation 3.4.7 of the University of Edinburgh, I hereby declare that the work presented in this thesis is my own and contribution from other workers is clearly indicated.

Robin L. Gilbert

QUOTATIONS

"The most incomprehensible thing about the world is that it is comprehensible".

"A theory can be proved by experiment; but no experiment leads to the birth of a theory".

Albert Einstein (1879-1955)

"One man's hyperactive mind makes no sense to another, unless tuned-in to the same wavelength; or one man's thoughts are another man's confusion"-
conclusion reached after having thesis drafts read by others.

(Robin L. Gilbert 1966-)

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ABSTRACT

Adenosine 3',5'-cyclic monophosphate (cAMP) analogues have antiproliferative effects on a range of cancer cells including breast, colon and lung. The most potent analogue, described to date, is 8-chloro cAMP but its mechanism of inhibition is poorly understood. The aim of this thesis was to investigate the mechanism of action of cAMP analogues on human cancer cell lines, in particular the hormone-responsive breast cancer cell line MCF-7.

Growth inhibitory effects of 8-chloro cAMP was confirmed on MCF-7 cells. However, this effect was not present in serum-free conditions and the degree of inhibition was dependent on the concentration of foetal calf serum in the medium. This effect could also be demonstrated in the presence and absence of added oestradiol suggesting that 8-chloro cAMP does not antagonise the oestradiol stimulation of MCF-7 cells. Effects of 8-chloro cAMP were also tested on other cancer cell lines derived from breast, ovary and colon. Inhibition of cellular proliferation was again dependent on the addition of serum in the culture medium. Partial characterisation and purification of serum was attempted in order to identify the component(s) responsible for this serum-dependent 8-chloro cAMP inhibition. The component(s) was heat labile and fractions separated by gel filtration chromatography containing inhibitory activity were eluted before the major albumin peak. When medium containing 8-chloro cAMP and serum was pre-incubated, antiproliferative activity remained after removal of most of the serum components by ultrafiltration. In serum-free conditions, phosphodiesterases conferred the ability to 8-chloro cAMP to inhibit growth of MCF-7 cells; addition of a phosphodiesterase inhibitor appeared to protect the cells from the antiproliferative effect in serum containing medium. High pressure liquid chromatography of cAMP analogues incubated with serum or with gel filtration fractions detected a peak that had identical UV spectrum and a column retention time as an 8-chloro adenosine standard. The concentration of adenosine analogue formed correlated with the concentration of serum in the medium and the degree of growth inhibition. Purified 8-chloro adenosine was directly toxic to MCF-7 cells cultured in the presence and absence of serum.

The data in this thesis suggest that 8-chloro cAMP (and 8-bromo cAMP) is metabolised by serum borne phosphodiesterases and 5'-nucleotidases. The antiproliferative effects of cAMP analogues in this culture system appear to be mediated largely or entirely via generation of their toxic adenosine metabolites.

ABBREVIATIONS

A	adenine
AP	activator protein
8-Br	8-bromo
C	cytidine
cAMP	cyclic adenosine 3':5' monophosphate
CAP	catabolite activator protein
CAT	chloramphenicol acetyltransferase
8-Cl	8-chloro
CPM	counts per minute
CRE	cAMP response element
CREB	cAMP response element binding protein
CRP	cAMP receptor protein
DBcAMP	dibutyryl cAMP
DMBA	7,12-dimethyl-benz(a)anthracene
DMEM	Dulbecco's Modified Eagles Medium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
G	guanine
GAP	GTPase activator protein
G-protein	guanine nucleotide binding protein
³ H	tritium
HITS	Hydrocortisone, Insulin, Transferrin and Selenium (section 2.2.1)
HITS medium	tissue culture medium containing HITS
HPLC	high pressure liquid chromatography
IBMX	isobutylmethylxanthine
IC ₅₀	50% inhibitory concentration

IF-s	serum inhibitory factor(s)
IgG	immunoglobulin G
IP ₃	inositol triphosphate
kinase A	cAMP-dependent protein kinase
mRNA	messenger RNA
p21	21 kDA protein product of the <i>ras</i> gene
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PDA	piperazine diacrylamide
PDE	cAMP-dependent phosphodiesterases
PDGF	platelet derived growth factor
PRPP	5-phosphoribosyl-1-pyrophosphate
RI	type I kinase A regulatory subunit
RII	type II kinase A regulatory subunit
SDS	sodium dodecyl sulphate
T	thymine
TEMED	N,N,N',N'-tetraamethylethylenediamine
Tris	Tris[hydroxymethyl]aminomethane

CONTENTS

INTRODUCTION	1 - 38
Diagram 1	9
Diagram 2	21
<u>SECTION 1: MATERIALS</u>	39-45
1.1 RADIOCHEMICALS	40
1.2 GEL ELECTROPHORESIS	40
1.3 ANTIBODIES	41
1.4 GENERAL CHEMICALS	41-42
1.5 OTHER REAGENTS	42-43
1.6 AUTORADIOGRAPHY	43
1.7 CELL LINES	44
1.8 TISSUE CULTURE	44-45
1.9 NON-CHEMICALS	45
<u>SECTION 2: METHODS</u>	46-64
2.1 TISSUE CULTURE	47-51
2.2 BUFFERS	52-53
2.3 PREPARATION OF CELL EXTRACTS	54-55
2.4 BRADFORD ASSAY	55
2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS	55-58
2.6 WESTERN BLOTTING	58-59
2.7 PHOTOAFFINITY LABELLING	59
2.8 AUTORADIOGRAPHY	59-60
2.9 CHARCOAL STRIPPING SERUM	61
2.10 DNA ANALYSIS BY FLOW CYTOMETRY	62
2.11 FILTRATION TECHNIQUES	62-64
<u>SECTION 3: RESULTS</u>	65-167
3.1 PARTIAL CHARACTERISATION OF THE MCF-7 CELL LINE	66-68
Figure 1(a)	69
Figure 1(b)	70
3.2 8-CI cAMP GROWTH INHIBITION OF MCF-7 CELLS IN CULTURE	71
Figure 2	72
3.3 THE EFFECT OF SERUM ON THE INHIBITORY EFFECT BY 8-CI cAMP WITH MCF-7 CELLS	73-74
Figure 3	75
Table 1	76
3.4 EXTRACTION OF PROTEIN FROM CELLS	77-78
Figure 4	79
3.5 QUANTITATIVE ANALYSIS OF p21 BY WESTERN BLOTTING	80-81
Figure 5	82
Plate 1	83

3.6	ALTERNATIVE METHODS OF QUANTIFYING WESTERNS	84-90
	Plate 2	91
	Plate 3	92
	Table 2	93
	Plate 4	94
	Plate 5	95
3.7	EFFECT OF 8-Cl cAMP ON THE EXPRESSION LEVEL OF p21	96-97
	Plate 6	98
	Table 3	99
3.8	THE EFFECT OF 8-Cl cAMP ON THE EXPRESSION LEVELS OF THE cAMP-DEPENDENT PROTEIN KINASE REGULATORY SUBUNITS	100
	Plate 7	101
	Table 4	102
3.9	CELL CYCLE ANALYSIS OF MCF-7 CELLS TREATED WITH 8-Cl cAMP	103-104
	Table 5	105
	Figure 6	106
3.10	SERUM-DEPENDENT 8-Cl cAMP INHIBITION OF MCF-7 CELLS	107
	Figure 7	108
3.11	USE OF SERUM-FREE MEDIUM TO STUDY THE INVOLVEMENT OF A PUTATIVE SERUM FACTOR DEPENDENT 8-Cl cAMP INHIBITION	109 - 112
	Figure 8	113
	Figure 9(a)	114
	Figure 9(b)	115
	Figure 9(c)	116
3.12	OTHER CELL LINES HAVE A SERUM DEPENDENCE FOR 8-Cl cAMP INHIBITION	117 - 118
	Figure 10	119
3.13	THYMIDINE UPTAKE ASSAY	120
	Figure 11	121
3.14	CONCENTRATION DEPENDENCE OF THE RESPONSE TO 8-Cl cAMP AND 8-Br cAMP	122
	Figure 12	123
	Figure 13	124
3.15	HEAT INACTIVATION OF THE SERUM-DEPENDENT 8-Cl cAMP INHIBITORY RESPONSE	125
	Figure 14	126
3.16	MOLECULAR WEIGHT ESTIMATION OF IF-s USING MOLECULAR WEIGHT CUT OFF FILTERS	127 - 128
	Figure 15	129
	Plate 8	130

3.17 GEL FILTRATION CHROMATOGRAPHY OF CONCENTRATED FCS	131 - 132
Figure 16	133
Plate 9	134
Figure 17	135
3.18 THE PRESENCE OF IF-s IS NOT REQUIRED DURING THE PERIOD OF CELL CULTURE FOR INHIBITION BY 8-Cl cAMP	136 - 137
Figure 18	138
3.19 EFFECT OF IBMX ON 8-Cl cAMP INHIBITION OF MCF-7 CELLS CULTURED IN HITS MEDIUM CONTAINING 10% FCS (V/V)	139 - 140
Figure 19	141
3.20 EFFECT OF PDE ON MCF-7 CELLS CULTURED IN HITS MEDIUM WITH 8-Cl cAMP	142
Figure 20	143
3.21 HPLC ANALYSIS FOR 8-Cl cAMP METABOLITES	144 - 146
Chromatogram 1	147
Figure 21	148
Figure 22	149
3.22 CONFIRMATION THAT THE METABOLITE PEAK IS 8-Cl ADENOSINE	150
Chromatogram 2	151
3.23 CONCENTRATION DEPENDENT FORMATION OF METABOLITE WITH cAMP ANALOGUES	152 - 153
Figure 23	154
3.24 EFFECT OF INCUBATION TIME ON METABOLITE FORMATION	155
Figure 24	156
3.25 IBMX INHIBITS THE GENERATION OF 8-Cl ADENOSINE FROM 8-Cl cAMP BY SERUM	157
Figure 25	158
3.26 THE EFFECT OF PDE ON METABOLISM OF 8-Cl cAMP IN SERUM-FREE MEDIUM	159 - 160
3.27 METABOLITE OF 8-Cl cAMP MEDIATED BY FCS FRACTIONS	161
Figure 26	162
Figure 27	163
3.28 METABOLISM OF 8-Cl cAMP BY OTHER SERA	164
Figure 28	165
3.29 INHIBITION OF THYMIDINE UPTAKE BY 8-Cl ADENOSINE	166
Figure 29	167
<u>SECTION 4: DISCUSSION</u>	168 - 185
4.1 HORMONE-RESPONSIVE MCF-7 CELLS	169 - 170
4.2 MECHANISM OF TAMOXIFEN ANTAGONISM	170 - 171

4.3	8-Cl cAMP ANTAGONISM OF OESTRADIOL STIMULATION IN MCF-7 CELLS	171-172
4.4	p21, KINASE A REGULATORY SUBUNITS AND CELL CYCLE ANALYSIS	173
4.5	SERUM-DEPENDENT 8-Cl cAMP GROWTH INHIBITION	174
4.6	CHARACTERISATION OF IF-s	174-176
4.7	METABOLISM OF 8-Cl cAMP	176-177
4.8	ADENOSINE TOXICITY	178-180
4.9	PHOSPHODIESTERASES AND 5'-NUCLEOTIDASES	181-183
4.10	TOXICITY OF 8-Br ADENOSINE	183-184
4.11	SERUM-FREE CULTURE CONDITIONS	184
4.12	THE FUTURE OF SITE-SELECTIVE cAMP ANALOGUES	184-185
SECTION 5: BIBLIOGRAPHY		186-211
LIFE IN THE LABORATORY		212

INTRODUCTION

Introduction:

Cancer can currently be clinically approached by a number of treatments, such as use of chemotherapeutic agents, radiation therapy, and combination of both. These treatments typically reduce the growth rate of the cancer, and in most part kill the cancer cells. A major disadvantage to these treatments, however, is the fact that they are harmful to normal cells, which limits the extent of treatment and may cause incomplete elimination of the disease. On the other hand, therapy targeted directly towards the cancer cell would improve the effectiveness of the treatment and increase tolerance by the patient. For example, some cancers, such as hormone-responsive breast cancer, can be treated with chemotherapeutic drugs designed to antagonise the hormone responsiveness of the cancer. Thus, a detailed understanding of the factors involved in regulation of proliferation may help to identify differences between normal cells and cancer cells (if such differences exist) and enable design of more effective agents for chemotherapy.

cAMP, a mediator of hormone signals (Sutherland & Rall, 1975) has been considered to play a role in cell growth and differentiation. The potential use of cAMP as a chemotherapeutic drug has recently been realised since the synthesis of specific cAMP analogues, which appear to have a broad spectrum of growth inhibition on a variety of cancer cells (Katsaros *et al.* 1987). The selectiveness of these agents for cancer cells and their mechanism of growth inhibition is currently under investigation. A discussion of factors involved in proliferation and the current understanding of cAMP and cAMP analogues' growth regulatory mechanism will be dealt with during the course of this introduction.

Factors Required For Culturing Cells:

The complexity of a multicellular organism makes it difficult to study factors involved in cellular proliferation. However, established cell lines, many of which are derived from cancer tissue, are capable of being cultured in-vitro, thus enabling closer analysis of key components required for growth. These cell lines are cultured in nutrient medium containing essential amino acids, nucleic acids, and carbohydrates, however, these ingredients alone do not maintain cellular proliferation, but require specialised additives for continued growth. Many of these specialised additives are supplied through the addition of serum, such as horse, calf, or foetal calf to the nutrient medium.

The 3T3 mouse fibroblast line was one of the first established cell lines in culture (Todaro & Green, 1963). When these cells are seeded at low density, they have morphology resembling the primary cells used to establish the cell line. In addition to having many of the primary cells' growth characteristics, though enhanced, 3T3 cells frequently have abnormal chromosome numbers. These "normal" cells grow in culture to form a monolayer of cells and become growth arrested due to contact with neighbouring cells.

The concept that multiple factors are required to be added to the medium for optimum growth came initially from the observation that 3T3 cells grow better in animal sera obtained from clotted blood (serum) rather than plasma (Ross *et al.*, 1974, Kohler & Lipton, 1974). It was found that a factor released from platelets gave optimum growth. This platelet derived growth factor, when added alone to serum-free medium, is a poor mitogen for 3T3 cells, however, optimum growth is possible on addition of

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plasma. In recent years many of these serum borne growth stimulating agents have been isolated and characterised.

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Hormones And Growth Factors:

Factors that control cellular proliferation and cellular metabolism can be grouped under two general headings: steroid-like hormones and non-steroid factors.

Steroid hormones are hydrophobic molecules that can directly cross the plasma membrane into the cytosol, and a cellular response to the hormone is dependent on the cell possessing the hormone's specific receptor protein. Binding of the hormone to its receptor results in a conformational change in receptor structure, thus exposing a DNA binding domain, which allows the receptor to directly interact with specific DNA sequences. Many of these receptors have been cloned, and DNA sequence analysis has confirmed a strong homology between the different types of receptors, especially in the hormone binding and DNA binding regions (Reviewed by Gehring, 1987). There are, however, small sequence differences between these homologous regions which are enough to confer ligand specificity for each receptor and interaction with specific DNA sequences (Green & Chambon, 1987; Klock *et al.*, 1987). Binding of the activated steroid receptor complex (ligand-receptor) to chromatin results in transcriptional regulation of a variety of genes. For example, the steroid hormone oestrodiol can induce expression of a large number of genes, many of which are involved in DNA replication, including DNA polymerase, thymidine kinase, and dihydrofolate reductase (Lippman, 1980).

Physiological concentrations of oestradiol (10^{-8} - 10^{-9} M) can stimulate growth of some cancer cell lines, including the hormone-responsive breast lines MCF-7 (Lippman *et al.*, 1976), ZR-75-1 (Darbre *et al.*, 1983), and the ovarian carcinoma lines PEO1 and PEO4 (Langdon *et al.*, 1990). In contrast, retinoids can be growth inhibitory to these types of cells (Ueda *et al.*, 1980, Lacroix & Lippman, 1980). There is, however, accumulating evidence to support the thesis that hormones are not mitogens, but induce the expression of genes which code for products that are mitogens. Secretion of these products by the cell can then directly stimulate adjacent cells in a paracrine response or directly feed back on the secreting cell in an autocrine response (Sporn & Torado, 1980). Many of these growth stimulating factors have been found secreted from human breast cancer cells after addition of oestradiol (Bates *et al.*, 1986; Bronzert *et al.*, 1987; Dickson *et al.*, 1986; Huff *et al.*, 1986).

These non-steroid factors, such as those secreted from oestradiol stimulated cells, are small hydrophillic polypeptides with molecular weights ranging from 1 to 40 kDa. Unlike hormone receptors, these proteins are unable to permeate the plasma membrane directly, but bind to specific receptor proteins situated within the plasma membrane with the ligand binding site exposed on the extracellular side. These receptors are anchored in the membrane and, therefore, require an alternative system to convey the signal from the plasma membrane to trigger cellular proliferation.

Signal Transduction:

Growth inducing factors regulate cellular metabolism through a variety of transmembrane signalling mechanisms, which involve a series of protein-protein interactions at the site of the plasma membrane. These protein interactions result in the generation of a second messenger product which is mobile in the cytosol.

Cyclic adenosine monophosphate (cAMP) is the best characterised second messenger to date (Reviewed by Gilman, 1987). Ligand (e.g., acetylcholine, adrenaline) binding to specific receptors produces an activated receptor complex which then interacts with membrane bound small guanine nucleotide binding proteins (G-protein). This interaction results in the exchange of GDP for GTP bound to the G-protein, thus activating the protein. However, this activation is very short lived due to the intrinsic GTPase activity of the protein. Activated G-protein can interact with the effector protein, adenylyl cyclase, also located on the membrane, which catalyses the conversion of ATP to cAMP. cAMP continues to be generated as long as activated receptor remains coupled to adenylyl cyclase via its G-protein. The elevation in cAMP concentration in the cytoplasm is transient due to cAMP-dependent phosphodiesterases, which convert cyclic AMP into inactive AMP. cAMP is the mobile second messenger which can bind to the regulatory subunits of cAMP-dependent protein kinases resulting in enzyme activation. These activated kinases phosphorylate specific proteins, which can lead to functional changes such as alteration in enzyme activity.

The above explanation of a G-protein describes the G_s protein (stimulatory), however, the G_i protein (inhibitory) when activated (e.g.,

ligand bound to muscarinic-type II acetylcholine receptor) inhibits the activity of adenylyl cyclase (Matesic *et al.*, 1991). Other G-proteins have been implicated in the regulation of K⁺ channels, Ca²⁺ channels (Yantani *et al.*, 1990), and phospholipase C (Smrcka *et al.* 1991) and many have still to have receptor and effector identified.

Another well characterised intracellular signalling pathway is the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Berridge & Irvine, 1984; MacPhee *et al.*, 1984; Davis & Czech, 1985). Activated receptor (e.g., bombesin bound to its receptor) couples to the Gq protein, which then activates the membrane bound enzyme phospholipase C (PLC) (Smrcka *et al.*, 1991). PLC hydrolyzes the membrane component phosphatidylinositol 4,5-bisphosphate, which generates two products inositol (1,4,5) triphosphate (IP₃) and diacylglycerol (DAG). IP₃ is soluble in the cytoplasm and is involved in increasing intracellular free Ca²⁺ ions. DAG transiently activates the calcium-dependent, lipid-dependent protein kinase C. Like cAMP, IP₃ and DAG are short lived messengers, since they are rapidly hydrolyzed by enzymes to inactive products. A similar system has also been suggested for the hydrolysis of phosphatidylcholine (Besterman *et al.*, 1986; Roscoff *et al.*, 1988).

Many of the growth factors, identified to date, appear to have receptors with intrinsic tyrosine-specific protein kinase activity, for example, epidermal growth factor (EGF) receptor, PDGF receptor, and fibroblast growth factor (FGF) receptor (Sherr *et al.*, 1985; Akiyama *et al.*, 1986). The tyrosine kinase is situated at the opposite end from the extracellular receptor site within the cytoplasm. This is activated on ligand binding to the receptor site and appears to autophosphorylate the

receptor. One of the consequences of this phosphorylation is a change in conformation of the cytoplasmic domain of the receptor (Keating *et al.*, 1988). In this phosphorylated state, the receptor can physically interact with cytoplasmic proteins that may be important in signal transduction (Kazlauskas *et al.*, 1990; Morrison *et al.*, 1989). Phosphatidylinositol-3 kinase, for example, associates with the tyrosine phosphorylated PDGF receptor resulting in the activation of this enzyme (Escobedo *et al.*, 1991). Many of the substrates for tyrosine phosphorylate receptors have still to be identified. Diagram 1 (page 9) schematically represents some of these signal transduction pathways.

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All components involved in signal transduction, the kinases that are activated and kinases' substrates, are still poorly characterised. Identification of the components of signal transduction and their function is further complicated, since second messenger systems do not appear to work in isolation of other pathways. Evidence has suggested that one second messenger system can interact with the components of other second messenger systems, consequently affecting the response of that system (Huganir & Greengard, 1987; Hall *et al.*, 1990). It has been shown, for example, that phosphorylation of the nicotinic acetylcholine receptor is catalyzed by at least three different protein kinases, cAMP-dependent protein kinase, protein kinase C, and a tyrosine-specific protein kinase (Huganir *et al.*, 1984). Phosphorylation of this receptor by cAMP-dependent protein kinase, however, increases the rate of desensitization which reduces the degree of response to acetylcholine (Huganir *et al.* 1986). Furthermore, Qureshi *et al* (1991) demonstrated that expression of a primary response gene in 3T3 cells requires the interaction of tyrosine kinase and kinase C. In addition to kinase effects, regulation of G-protein

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coupling to effector has also been suggested. Yatani *et al.* (1990) suggest from their data that the p21.GTP-GAP complex can interfere with the coupling of the G-protein to K⁺ ion channels, thus preventing the channels from opening. Many other signal transduction pathways are only now being elucidated, such as the insulin receptor, prostaglandins, arachidonic acid, and phospholipase A₂. Their interference with other pathways has yet to be considered.

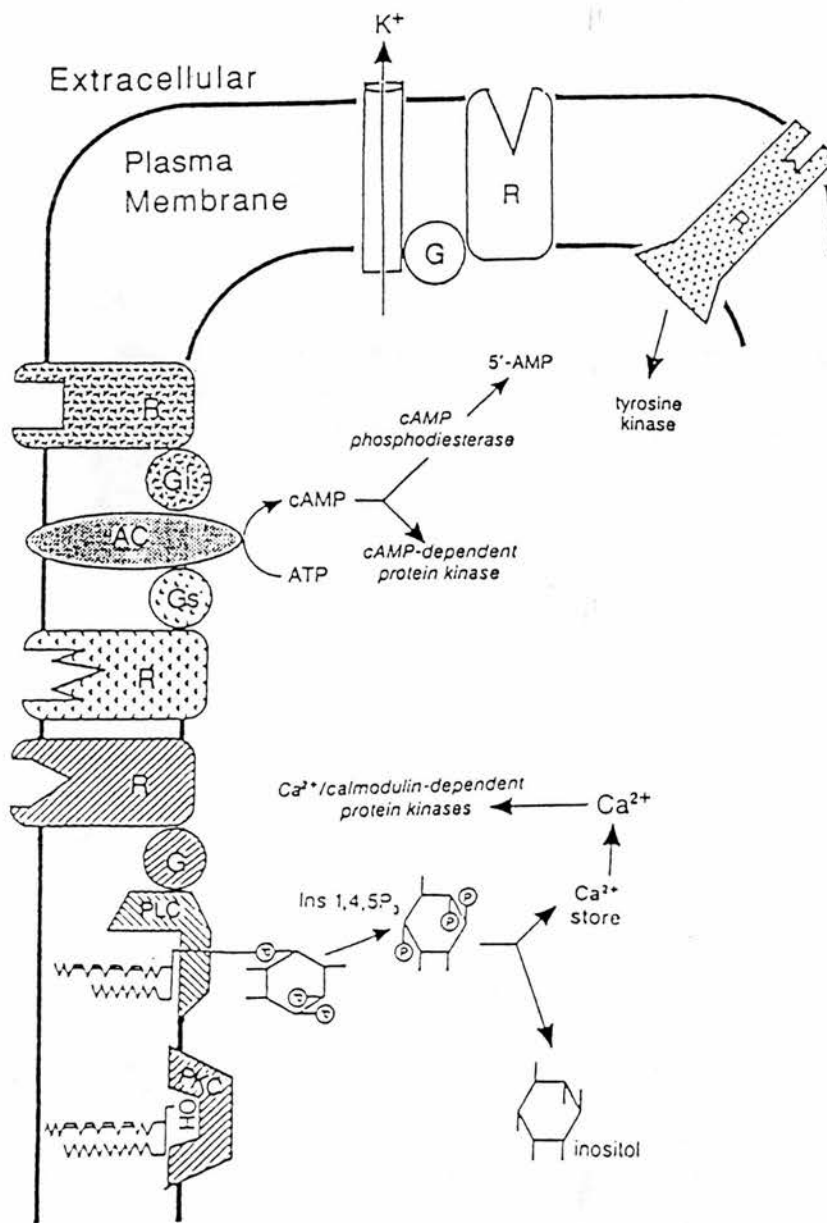


Diagram 1:

Schematic representation of some signal transduction pathways.

R- receptor; **G-** G-protein; **Gi-** inhibitory G-protein;

Gs- stimulatory G-protein; **AC-** adenylate cyclase;

PLC- phospholipase C; **PKC-** protein kinase C.

Oncogenes:

Due to the complexity of signal transduction mechanisms, especially the study of growth factor systems, virus induced tumour models have been employed to identify the key components required for control of proliferation. Identified viruses that can transform cells in culture into a cancerous state include SV40, polyoma, and papilloma viruses (Bishop, 1985). Analysis of the viruses' genetic material has identified genes, generally known as oncogenes, responsible for the transforming principle. Many of these viral oncogenes appear to have strong DNA sequence homology with host genes, so-called proto-oncogenes, which have the potential to become oncogenes via mutation.

One of the first viral oncogenes recognised was *ras* oncogene, which is responsible for the transforming principle of the Harvey and Kirsten strains of rat sarcoma virus (Harvey, 1964; Kirsten & Mayer, 1967). Proto-oncogenes of *ras* have also been widely identified in eukaryotic cells and since the identification of transforming *ras* genes in human tumours (Reviewed by Marshall, 1988; Barbacid, 1987), the study of *ras* has become an area of intense research.

Ras genes code for 21 kDa membrane bound proteins (p21) that have striking homology with G-proteins and possess intrinsic GTPase activity. Many of the transforming p21 products appear to have a defective GTPase. Hydrolysis of GTP by these transforming proteins is either impaired or non-existent, resulting in prolonged activation of p21 (McGrath *et al.*, 1984; Sweet *et al.*, 1984; Willumsen *et al.*, 1986; Lacal *et al.*, 1986). The striking similarities between p21 and G-proteins have led researchers to postulate that p21 is involved in signal transduction. However, the

receptor that interacts with p21 and its downstream effector have still to be elucidated. A possible effector for p21 is currently being investigated since the discovery of GTPase Activating Protein (GAP) (Trahey & McCormick, 1987).

Even though the role of p21 in signal transduction is not understood completely, evidence has strongly implicated p21 in the role of replication. As was shown by Stacey & Kung (1984), the mouse fibroblast NIH/3T3 cells can be transformed by microinjecting viral p21. Using this model, Mulcahy *et al.* (1985) demonstrated that microinjecting anti-p21 antibody during DNA replication (S phase) does not prevent NIH/3T3 cells from completing replication. However, injection of anti-p21 antibody before the start of S phase prevents the cell from initiating DNA replication. Since the antibody can prevent initiation of S phase in the presence of serum, the involvement of p21 in the initiation of DNA replication is strongly implied. Moreover, the relevance of p21 in cellular proliferation is corroborated by the observed incidence of *ras* gene mutations in human tumours, including colorectal cancer (Bos *et al.*, 1987), acute myeloid leukaemia (Farr *et al.*, 1988), and pancreatic carcinoma (Almoguerra *et al.*, 1988) of 25 - 50% or higher.

Like *ras* oncogenes, other oncogenes identified appear to have products that are also involved in some aspect of signal transduction. One such oncogene is *v-sis* which has strong homology with the β -chain of the mitogen PDGF and can transform cells that express the PDGF receptor (Doolittle *et al.*, 1983; Gazit *et al.*, 1984). *erb-B*, another example, has striking homology with the EGF receptor, but does not bind EGF and has a constitutively active tyrosine-specific protein kinase (Downward *et al.*, 1984). In addition to *erb-B*, there are other oncogenes such as *src*, *fms*,

and *lck* which code for products that also have tyrosine kinase activity and are situated at the membrane (Jove & Hanafusa, 1987; Sherr *et al.*, 1985; Sefton, 1991). There are also oncogenes which code for products that are located within the nucleus. Two such examples are *myc* and *fos* whose products, when expressed in the presence of PDGF, have implicated their importance in mitogenesis (Eisenman *et al.*, 1985; Kelly *et al.*, 1983; Cochran *et al.*, 1984).

Controlling Proliferation Via Signal Transduction Pathways:

In light of the important role signal transduction plays in regulating growth of cells, these pathways can offer a potential target for controlling proliferation, especially with a view to regulating the growth of cancer cells. So far, a number of approaches have already been implemented based on the understanding of steroid hormones and growth factors. Tamoxifen, for example, is an oestrogen antagonist and has proved to be a valuable drug in the treatment of hormone-responsive cancers. It prevents oestradiol from activating its receptor, thus inhibiting the expression of mitogenic agents. Other approaches have been directed towards using growth factor antagonists and the use of antiproliferative agonists, such as the factor TGF- β . Other methods being considered involve regulating the expression level of oncogene products and introducing second messengers involved in an antiproliferative effect.

Cell Cycle:

It is important to understand that there are several stages to cellular proliferation including DNA replication (S phase), mitosis and cytokinesis (M phase) At each of these phases specific biological components are required. Second messengers, produced in response to extrinsic factors, induce a transient activation of key specific proteins, many of which may only exist during a particular phase of the cell cycle. Established cell lines have cell cycles with specific periods of time for each phase, which can be manipulated by factors in the culture medium. Alteration in the length of the cell cycle usually occurs in the phases between mitosis (M phase) and DNA replication (S phase), known as G1 phase, and between S phase and M phase, known as G2 phase. This is perhaps the simplest cell cycle known. Another type of cycle is one associated with cells that are quiescent due to starvation of various essential nutrients or due to confluence. Cells that lack essential components, such as a growth factor or an essential amino acid, stop cycling in the G1 phase in order to prevent premature arrest during chromosomal replication in S phase (Prescott, 1976). This arrested state is known as Go and is associated with a reduction in protein synthesis (Austin & Clemens, 1981) and an increase in cAMP content (Boynton & Whitfield, 1979). Addition of the missing component to starved cells can bring about a transition from Go to G1 by reversing changes induced during starvation, including initiating protein synthesis and reducing cAMP content (Rochette-Egly *et al.*, 1979; Whitfield *et al.*, 1982). Some cells, such as lymphocytes, hepatocytes, and pancreatic acinar cells, have a different type of Go than the one described for quiescent cells starved of

nutrients or confluent in culture. These particular cells retain protein synthesis and have very little or no proliferation-related components (Baserga, 1976). When stimulated, specific cell cycle genes are transcribed, and some protein synthesis activity associated with the G₀ phase are suppressed (Walker & Whitfield, 1981; Baserga, 1981). In order to understand how second messenger systems can increase the rate of proliferation, it would be necessary to identify the components involved in these pathways during the different phases of the cell cycle. The observation that quiescent cells have elevated cAMP levels and the fact that chronic exposure of endogenous cAMP can cause growth inhibition led to the hypothesis that cAMP was a negative regulator of growth. This hypothesis was overwhelmingly supported due to numerous reports detailing the inhibitory effects of cAMP derivatives and cAMP-elevating agents (Reviewed by Ryan & Heidrick, 1974; Pastan *et al.*, 1975). There is, however, an alternative hypothesis that cAMP is a positive regulator of proliferation (Reviewed by Boynton & Whitfield, 1983).

cAMP As A Positive Regulator:

In 1961, Selye *et al.* reported that exposure of the cAMP elevating agent β -adrenergic catecholamine to growth inactive acinar cells induced a massive proliferative response. In addition, MacManus & Whitfield (1969) reported that low concentrations of cAMP (10^{-8} - 10^{-6} M) stimulated DNA synthesis of rat thymic lymphocytes. However, addition of higher cAMP concentrations inhibited proliferation. cAMP and cAMP elevating compounds have also been demonstrated as positive regulators in cells from other tissues including liver, kidney, breast, and skin

(Reviewed by Boynton & Whitfield, 1983).

Pancreatic acinar cells stimulated by the synthetic β -adrenergic catecholamine isoproterenol display two cAMP surges. The first peaks after 10 to 15 minutes and the second 6 to 8 hours before the onset of DNA synthesis (Durham *et al.*, 1974; Guidotti *et al.*, 1972). Inhibition of the second cAMP surge by propranolol 8 hours after isoproterenol addition prevents initiation of DNA synthesis (i.e., entry into S phase), an effect that can be overcome by the introduction of dibutyryl-cAMP (DBcAMP) and theophylline (a phosphodiesterase inhibitor) (Tsang *et al.*, 1980). These cAMP surges have also been identified in other cells that respond to cAMP as a positive regulator, and again the cAMP surge before S phase appears to be important for initiation of DNA synthesis. However, if the fall of cAMP in liver cells is prevented at the point of entering S phase, DNA synthesis is inhibited (Boynton & Whitfield, 1979). These data suggest that this second cAMP surge is required to initiate DNA synthesis and that degradation of the cAMP is required for DNA synthesis to proceed. This data could explain observations by other workers that high concentration of cAMP or cAMP analogues present throughout the length of the cell cycle can inhibit cellular proliferation.

cAMP As A Negative Regulator:

Negative regulation of cellular proliferation by cAMP, cAMP-derivatives, and cAMP-elevating agents, though possibly not the normal physiological role of cAMP in the cell cycle, offered a new approach to controlling proliferation of cells, especially cancer cells. Ryan & Heidrick (1968) observed that addition of cAMP (300 μ M) to the culture medium of

tumour cells, including HeLa, HEp-2, and F1 amnion lines, resulted in a growth inhibition of up to 89% after an incubation period of four days. This inhibition appeared to be dose-dependent and non-cytotoxic, since cells resumed proliferating after removal of the second messenger. Furthermore, addition of the cAMP metabolites, 5'-adenosine monophosphate and adenosine produced no effect on cellular proliferation in their culture system (Heidrick & Ryan, 1970). These observations of inhibitory growth response were extended to *in-vivo* studies with tumour models, such as Walker 256 and MTW9 mammary carcinoma, and 5123 hepatoma. cAMP analogues, such as N⁶, O^{2'}-dibutyryl-cAMP (DBcAMP) and 8-Br cAMP, proved to be more efficacious than cAMP on inhibition of cellular proliferation (Cho-Chung, 1974). The effectiveness of the analogues over cAMP at equimolar concentrations might be explained by their increased resistance to hydrolysis by phosphodiesterases (Moore *et al.*, 1968).

Tumour Regression Associated Protein:

DBcAMP can inhibit growth of xenografts of the 7,12-dimethylbenz(a)anthracene (DMBA) induced hormone-responsive mammary carcinoma (Cho-Chung & Gullino, 1974; Huang & Cho-Chung, 1982) which is also growth inhibited by oestrogen ablation (ovariectomy) (Huggins *et al.*, 1961). Cho-Chung & Redler (1977) attempted to understand the mechanism of DBcAMP growth inhibition by studying nuclear protein phosphorylation from isolated tumour nuclei, and identified a nuclear protein that was phosphorylated in tumour treated with DBcAMP. The phosphorylation of this protein disappeared on removal of DBcAMP or addition of oestradiol (growth stimulatory) to the ovariectomized mice. Furthermore,

cytoplasmic cAMP binding and protein kinase also appeared to translocate to the nucleus in regressing tumour (treated with DBcAMP) (Cho-Chung, 1974; Cho-Chung & Clair, 1977). This accumulation of protein kinase in the nuclei was previously reported from rat liver (Palmer *et al.*, 1974; Castagna *et al.*, 1975) and calf ovary tissue (Jungmann *et al.*, 1974) treated with cAMP, which led to the suggestion that a connection existed between the mechanism of steroid growth stimulation and cAMP induced growth inhibition. Cho-Chung & Redler (1977) concluded from these data that this regression-associated protein may be the regulatory subunit of cAMP-dependent protein kinase.

cAMP-Dependent Protein Kinase:

cAMP-dependent protein kinase (kinase A) is activated by the second messenger cAMP which then leads to protein phosphorylation. The inactive form of kinase A is a tetramer composed of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits, of which there are two binding sites per subunit, result in the dissociation of the catalytic subunits from the regulatory dimer. The free catalytic subunits are in an active state. Kinase A phosphorylate serine residues, and the enzyme pyruvate kinase is a known substrate. The amino acid sequence of pyruvate kinase was reported by Zetterqvist *et al.* (1976), and this sequence was used to derive a synthetic peptide substrate for cAMP-dependent protein kinase, Kemptide (Kemp *et al.*, 1976). Other substrates for kinase A include phosphorylase kinase and triacylglycerol lipase. The ability of the catalytic subunit of kinase A to phosphorylate a serine residue depends not only on the accessibility due to protein conformation,

but also due to the surrounding amino acid sequence. The sequence homology for potential serine phosphorylation appears to contain two basic amino acids on the amino terminus end of the serine, one of which is arginine, for example, Leu-Arg-Arg-Ala-Ser-Leu-Gly (pyruvate kinase, Kemptide). The phosphorylation of enzymes by kinase A can result in the modulation of their activity.

In most mammalian tissues there appear to be two types of kinase A which were originally identified by their different retention characteristics to ion-exchange chromatography columns (Krebs, 1972). cAMP-dependent kinase activity eluted from the column with low salt was termed type I and the second, with a higher salt concentration termed, type II. In addition, these different kinase A types also have different binding affinities for cAMP, however, both types appear to have identical catalytic activities (Beebe & Corbin, 1986; Weber *et al.*, 1982). Distinction between type I and type II resides in differences between the regulatory subunits (R) which have approximate molecular weights of 49 kDa (RI) and 55 kDa (RII), respectively. The use of molecular biology has identified four different mRNAs that code for regulatory subunits RI $_{\alpha}$ (Lee *et al.*, 1983), RI $_{\beta}$ (Clegg *et al.*, 1988), RII $_{\alpha}$ (Scott *et al.*, 1987) and RII $_{\beta}$ (Levy *et al.*, 1988). Three isoforms of the catalytic (C) subunit have also been identified: C $_{\alpha}$ (Uhler *et al.*, 1986), C $_{\beta}$ (Uhler *et al.*, 1986) and C $_{\gamma}$ (Beebe *et al.*, 1990). Relative expression of each of these subunits varies in different tissues, but preferential co-expression of either one of the regulatory subunits with the catalytic subunits has not been found (Showers & Maurer, 1986).

The function of the two types of protein kinase A have still to be

fully explored. Kinase A exists in tissues as a mixture (Corbin *et al.*, 1975; Hofmann *et al.*, 1975), and the relative levels of each type could confer a different response of the cell to cAMP. For example, high expression of type I in a cell, which has a higher affinity for cAMP compared to type II, could confer a greater kinase A activation for the same level of cAMP compared with a cell containing higher expression of type II. This would imply that cells expressing more type I than type II are more sensitive to the presence of a ligand that produces cAMP as its second messenger.

Site-Selective cAMP Analogues:

Early studies involving the use of high concentrations of cAMP and cAMP analogues were unable to determine the precise role of both types of protein kinase A in cellular proliferation, since at millimolar concentrations of cAMP or cAMP derivatives both regulatory subunits are saturated. The advent of site-selective cAMP analogues enabled a more detailed investigation into the role of each type of kinase in proliferation. The two binding sites for cAMP on each regulatory subunit are denoted as site 1 (or site B) and site 2 (or site A). Analogues that have the adenine ring modified at position C-2 or C-8 selectively bind site 1, and C-6 modified analogues selectively bind site 2, as defined by binding studies with purified subunit (Døskeland, 1978; Rannels & Corbin, 1980; see diagram 2 for structure of cAMP; page 21). Competition studies of two different analogues specific for the same binding site have shown that they only compete for that one binding site on the regulatory subunit and not the other. This competition is only minimally affected by addition of an analogue that has specificity for the opposite binding site. Moreover, C-

8-thio or halogen analogues preferentially bind to site 1 of type II rather than type I protein kinase (Ogreid *et al.*, 1985). These analogues are therefore not only specific for a specific binding site but for a regulatory subunit. Furthermore, the binding of an analogue to one site of a regulatory subunit can cooperatively enhance the binding of ligand to the opposite site. Therefore, addition of two site-selective analogues specific for opposite sites of the regulatory subunit can synergistically activate the protein kinase (Ogreid *et al.*, 1985). However, potent activation of the kinase is possible on addition of only one site-selective analogue (Kerlavage & Taylor, 1982). This activation is presumably due to the positive cooperativity that exists after a ligand has bound to one site, thereby enhancing the binding of endogenous cAMP or the analogue for the other site of the regulatory subunit. Alternatively, a single site-selective analogue may lead to partial dissociation of the protein kinase A holoenzyme; a stable complex of two bound ligands to protein kinase with one dissociated catalytic subunit has been identified (Connelly *et al.*, 1986). The synthesis of site-directed cAMP analogues enables the activation of specific kinase A types, thus facilitating investigation into the role each kinase A type has in cellular metabolism.

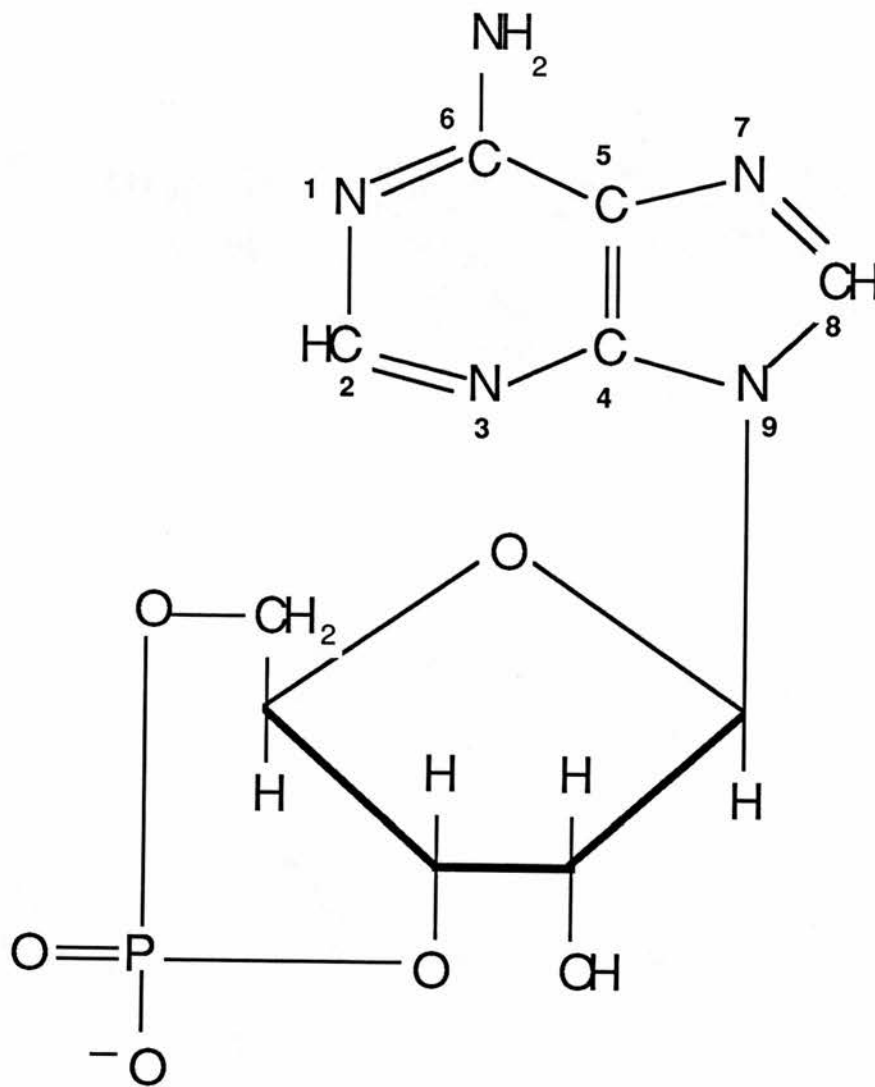


Diagram 2: Adenosine 3':5' cyclic monophosphate

Antiproliferative Effect of Site-Selective Analogues:

Many of the early studies with DBcAMP required millimolar concentrations of drug, and it was believed that many of the growth inhibitory effects were due to toxicity. Furthermore, many of the established cancer cell lines tested appeared to be resistant to DBcAMP. In contrast, a range of site 1 and site 2 selective cAMP analogues screened on a broad spectrum of established cancer cell lines, including breast, colon, and lung, proved more effective than DBcAMP (Katsaros *et al.*, 1987). The most potent analogue tested was 8-chloro cAMP (8-Cl cAMP) (C-8 analogue) which had a 50% inhibitory concentration (IC_{50}) on cancer cells in the range of 1 - 25 μ M after a four day exposure. The best C-6 analogue was N⁶-benzyl with an IC_{50} of 15 - 25 μ M. Moreover, the addition of phosphodiesterase inhibitors, such as isobutylmethylxanthine or theophylline, which when added alone had little effect on cellular proliferation, did not enhance the inhibitory effect when added with these cAMP analogues. This suggested that the toxic metabolites were not responsible for the antiproliferative effect by cAMP analogues (Tortora *et al.*, 1988; Cho-Chung *et al.*, 1989). From these data it was concluded that the concentrations required to produce growth inhibition were below levels at which degradation of the analogue by phosphodiesterase could take place. Furthermore, these analogues were considered to be non-cytotoxic, since cells treated for three days with 8-Cl cAMP (5 - 50 μ M) recovered and continued to proliferate after removal of the analogue. Growth inhibition with 8-Cl cAMP also appeared to be associated with a decrease in the level of the *ras* oncogene product, p21, however, it was not associated with a block in cell cycle progression (Katsaros *et al.*,

1988; Tagliaferri *et al.*, 1988). In contrast, addition of an 8-Cl cAMP metabolite, 8-Cl adenosine, led to the conclusion that it was toxic to cancer cells, since cancer cells exposed to equimolar concentrations of 8-Cl adenosine for three days did not resume proliferating after removal of the agent. Furthermore, a block in the cell cycle, between mitosis and DNA replication, was evident and a change in expression of *ras* p21 was not detected (Katsaros *et al.*, 1988; Tagliaferri *et al.*, 1988). From these data, the effects of 8-Cl cAMP did not appear to be mediated through its toxic adenosine metabolite. Interestingly, these site-selective analogues are specific inhibitors of transformed cell proliferation rather than normal cells. The growth inhibitory effect of these analogues on non-transformed NIH/3T3 cells was only 15 - 30% of the growth inhibition demonstrated in the transformed line (Cho-Chung *et al.*, 1989). At concentrations that effectively inhibit the transformed lines, these analogues have also been shown to produce little (less than or equal to 10%) or no inhibition on normal rat kidney cells (Tortora *et al.*, 1989), normal mammary epithelial cells, and normal blood lymphocytes (Cho-Chung, 1989). The micromolar concentrations of site-selective analogues required to inhibit a wide spectrum of cancer lines suggest that these site-selective cAMP analogues may be useful as chemotherapy agents in the prevention and cure of cancer. Using these more potent analogues compared to DBcAMP and cAMP investigators have studied the molecular effects in an attempt to understand the role of protein kinase A in cellular proliferation.

cAMP Analogues Antagonise The Hormone Response:

As previously discussed, addition of DBcAMP can mimic the response of oestradiol ablation in regression of the hormone-responsive DMBA-tumour (Cho-Chung & Redler, 1977); the increased phosphorylation of regression associated protein by cAMP was inhibited by addition of oestradiol (Cho-Chung & Doud, 1978). In the same system, it was also shown by Bodwin *et al.* (1978) that during regression of the tumour, induced either by DBcAMP or ovariectomy, cAMP-binding activity increased while oestrogen binding decreased in the cytosol and nucleus. Furthermore, when DBcAMP treatment was ceased, or oestrogen was injected into the mice, the opposite effect occurred: cAMP-binding activity decreased and oestradiol binding activity increased. These opposite effects between cAMP and oestrogen were not due to competition for each others binding proteins (Bodwin *et al.*, 1981). Alteration in the oestrogen binding level by cAMP was also demonstrated *in-vitro* using cultured endometrial and breast cells (Fleming *et al.*, 1984). In addition to these effects, cellular *ras* p21 was shown to decrease in regressing tumours after hormone withdrawal (Huang & Cho-Chung, 1984).

Site-selective analogues were used by Katsaros *et al.* (1988) to further investigate this apparent antagonism between cAMP and oestradiol in the hormone-responsive breast cancer cell line MCF-7. Addition of 17β -oestradiol (10 nM) to MCF-7 cells resulted in an increase in cellular proliferation of 2 to 3 fold over cells cultured in the absence of steroid. This oestradiol stimulated proliferation was reduced by 40% after three days with cells cultured in the presence of the oestrogen-antagonist tamoxifen (1 μ M). Addition of 8-Cl cAMP (10 μ M) in the presence of oestradiol resulted in a 70% growth inhibition of these MCF-7 cells after

three days. The C-6 analogue, N⁶-Benzoyl cAMP (20μM), proved to be as effective as tamoxifen (1 μM), however, combination of 8-Cl cAMP (1μM) and N⁶-Benzoyl cAMP (0.5μM) synergistically inhibited growth and stopped cellular proliferation after three days. Katsaros *et al.* (1988) also demonstrated that MCF-7 cells inhibited by 8-Cl cAMP (2 μM) for 9 days would eventually resume proliferating after drug removal. Addition of 8-Cl adenosine or 6-mercaptopurine at the same concentration and duration of exposure as 8-Cl cAMP not only inhibited cellular proliferation, but proliferation never resumed on removal of these agents.

ras p21 appears to play an important role in the control of proliferation, thus, regulation of *ras* p21 expression in tumours by oestradiol and cAMP may suggest a potential mechanism by which these agents regulate proliferation. Katsaros *et al.* (1988) investigated the effect of oestradiol and 8-Cl cAMP on the expression of two oncogenes, *myc* and *ras*. The protein products of the *myc* and *ras* oncogene remained elevated in MCF-7 cells treated with oestradiol and tamoxifen. 8-Cl cAMP (10 μM), however, reduced the expression levels of both oncogenes; *ras* p21 was reduced by 48% of the control after 3 to 4 days. Furthermore, expression of *ras* p21 was reduced by 90% of control MCF-7 cells treated directly with 8-Cl cAMP (10 μM) without the supplement of added oestradiol (Tagliaferri *et al.*, 1988). This reduction in *ras* p21 was not evident on addition of 8-Cl adenosine. From these data, it was concluded that the antagonism between tamoxifen and oestradiol was different from that of 8-Cl cAMP, since tamoxifen did not modulate expression of *c-myc* and *c-ras*.

Regulation of oncogenes could be a potential method of controlling proliferation of a cell, but precisely how oestrogen and cAMP control p21 expression is as yet unknown. Oestrogen appears to regulate the binding

levels of cAMP, and cAMP regulates the binding levels of oestradiol (Bodwin *et al.*, 1978; Fleming *et al.*, 1984). However, it is not known if oestrogen or cAMP can directly regulate *ras* oncogene expression. The level at which this antagonism takes place in the cell is also unclear. It could occur either at the site of the gene promoter or alternatively earlier on within the cytosol preventing the activation of a transcription complex. It is unlikely that cAMP modulates oestradiol regulated expression of *ras* p21 directly through antagonism alone, since cAMP analogues have been effective at altering expression levels in non-hormone responsive cell lines (Tagliaferri *et al.*, 1988).

Molecular Events Associated With cAMP Analogues:

Tagliaferri *et al.* (1985) previously demonstrated that the expression of *ras* p21 was inhibited in Harvey *ras* transformed mouse fibroblast cells exposed to DBcAMP or 8-Br cAMP. This decrease in *ras* p21 expression was concomitant with a change in the cell phenotype to one resembling a non-transformed cell. Furthermore, 8-Cl cAMP was shown to inhibit expression of p21 in non-hormone responsive breast cancer line MDA-MB-231 and the LS-174T colon carcinoma line (Tagliaferri *et al.*, 1988; Katsaros *et al.*, 1987). Ally *et al.* (1989) investigated the regulation of oncogene expression in the lung xenograft LX-1 treated with 8-Cl cAMP. In the first 6 hours of exposure, mRNA levels of *c-myc* had decreased by 50% of the control, whereas, the *N-ras* message level had increased by approximately two-fold. By seven days the level of both oncogene mRNA levels were equally reduced below the level of controls, thus indicating that the effect of 8-Cl cAMP on reducing *N-ras* mRNA level was not via direct modulation and probably required expression of other proteins to

affect the decrease. The initial increase in *N-ras* mRNA may have been a direct effect, since, after only one hour, the level had increased to greater than two-fold of the control. This may have been via increased gene expression or stability of *N-ras* mRNA.

In addition to the modulation of oncogenes, other biochemical changes have been reported in the presence of cAMP analogues. These changes include induction of differentiation in leukaemic cells (Tortora *et al.*, 1988) and suppressed production of transforming growth factor- α (TGF- α) in Kirsten *ras* transformed rat kidney fibroblast cells (Tortora *et al.*, 1989).

Leukaemic cells appear to have lost the ability to mature and instead continue to proliferate (Sachs, 1980). Tortora *et al.* (1988) demonstrated that 8-Cl cAMP could inhibit the growth of human leukaemic cell lines, including HL-60 (acute promyelocytic) and Molt-4 (acute T lymphocytic) between 5 - 20 μ M. These authors also determined that incubating HL-60 cells for three days with 8-Cl cAMP resulted in a marked increase in the expression of monocyte-specific surface antigens that are associated with maturation and a decrease in the markers associated with immature cells. In addition, loss of nucleoli and a decrease in nuclear-to-cytoplasmic ratio occurred, giving cells a phenotypic appearance similar to mature cells.

TGF- α is a potent mitogen for fibroblasts and epithelial cells, and its role has been considered in paracrine and autocrine growth of various cancer cells (Derynck, 1988). As demonstrated by Tortora *et al.* (1989), the production of TGF- α in 8-Cl cAMP growth inhibited Kirsten *ras* transformed rat fibroblast cells was suppressed, concomitant with a decrease in *ras* p21 and a reversion of the transformed phenotype.

Furthermore, it was observed that 8-Cl cAMP could prevent transformation of mouse mammary cells by TGF- α (Ciardiello *et al.*, 1990). Thus, there appears to be a multitude of biological changes that occur in cancer cells treated with cAMP analogues, all of which may be important factors in controlling proliferation.

Relative Expression Levels Of Type I and Type II

Kinase A:

The relative levels of the two types of kinase A have been investigated the use of a number of techniques, including chromatography, Scatchard analysis, antibodies, and photoactivated incorporation of 8-azidoadenosine 3',5'-cyclic [32 P] monophosphate (Corbin *et al.*, 1975; Sugden *et al.*, 1976; Hofmann *et al.*, 1977; Pomerantz *et al.*, 1975). Studies involving the use of these techniques on cancer cells revealed a predominance of RI over RII levels, and in some lines the only detectable subunit was RI (Katsaros *et al.*, 1987; Cho-Chung, 1989). These cell lines include those that have been previously discussed during the course of this introduction. Cells growth arrested or differentiated by addition of site-selective cAMP analogues appear to have a decreased level of RI and an increased level of RII (Katsaros *et al.*, 1987; Cho-Chung, 1989). The higher expression of RI correlates with proliferating cells, while RII correlates with growth inhibited and differentiated cells. Studies in primary tumours, however, are not as clear cut as for those of the cell lines. Nevertheless, there are many reported cases of tumours having a predominance of RI over RII. For example, a study by Eppenberger *et al.* (1980) compared primary breast tumours with normal breast and showed

significantly higher ratios of type I (RI) to type II (RII) protein kinase. Other studies for the same tissue type have revealed changes in the RI : RII expression ratio (Handschin *et al.*, 1983) as well as an increase in RI (Weber *et al.*, 1981). Moreover, a study of renal cell carcinomas measured an increase in the type I : type II ratio by two-fold (Fossberg *et al.*, 1978), and another in Wilm's tumours showed a two times greater ratio of RI : RII when compared to normal tissue (Nakajima *et al.*, 1984).

Alteration in the ratio of type I and type II (or RI and RII) expression does not appear to be a unique feature of cells with a malignant phenotype. The relative content of type I and type II varies among normal tissues (Corbin *et al.*, 1975; Sugden *et al.*, 1976; Hofmann *et al.*, 1977). Furthermore, developmental studies have also revealed that the relative expression of both types of protein kinase A may play an important role in tissue development and differentiation. Two studies in mouse heart development showed that the ratio of type I : type II decreased by three-fold in hearts from 7 or 14 day olds to those from mature adults (Malkinson *et al.*, 1978; Haddox *et al.*, 1979). These data suggest that RI is associated with the development phase of the mouse heart and RII with the increase in heart size. In rat testes the level of type I remained constant during development, whereas, a seven-fold increase in type II occurred between birth and 90 days of age (Lee *et al.*, 1976). This increase in type II coincides with the acquisition of the capacity for steroidogenesis and spermatogenesis. A study of ovarian follicle granulosa cell differentiation in hypophysectomised rats demonstrated that injections of oestradiol and follicle stimulating hormone induced differentiation (Jonassen *et al.*, 1982). These treatments resulted in an increased level by 10 to 20 fold of type II in the granulosa cells. Ventral prostate, another steroid responsive tissue, also appears to have an

increase in the levels of type I with a decrease in type II levels in the presence of steroids (Richards & Rolfes, 1980). These tissue studies and experiments with cell lines have led to the general view that RI (or type I protein kinase A) is involved in proliferation and that RII (or type II protein kinase A) is involved in growth arrest and differentiation.

Modulation Of The Regulatory Subunits:

Insight into the 8-Cl cAMP-induced regulation of cancer growth became clearer with the availability of antibodies specific for the different regulatory subunits. Ally *et al.* (1988) used specific antibodies for RI, RII $_{\alpha}$, and RII $_{\beta}$ to study the early events associated with addition of 8-Cl cAMP. Using the LS-174T colon carcinoma line, these authors observed a rapid translocation of the RII $_{\beta}$ subunit from the cytoplasm to the nucleus. This translocation was a very rapid event occurring in ten minutes. They also used a nuclear run-off transcription assay to determine the transcriptional activity of the regulatory and catalytic subunit genes on exposure of 8-Cl cAMP. After one hour there was no apparent change in the transcriptional activity of the catalytic or RII $_{\alpha}$ subunit. However, RI levels dropped to 50% after a one hour exposure to 8-Cl cAMP, and this change was not detectable in the first 30 minutes. Moreover, transcriptional activity of the RII $_{\beta}$ gene increased by two-fold after only 30 minutes. Interestingly, Alley *et al.* (1988) also found that 8-Cl cAMP, though it has a higher affinity for the RII subunit, had a three-fold greater activation of type I kinase A. Although, N⁶-butyryl cAMP has a higher affinity and activation for type I compared to 8-Cl cAMP, this analogue had little or no effect on proliferation of the LS-174T cells

(Katsaros *et al.*, 1987). These data suggest that kinase activity was not the determining factor for the cellular response of LS-174T cells, but that the $R_{II\beta}$ subunit was the key to the growth regulatory action of cAMP.

The involvement of the $R_{I\alpha}$ and $R_{II\beta}$ regulatory subunit has also been investigated with the use of antisense techniques. Antisense sequences, which are complementary to the mRNA sequence (either synthesized from DNA or RNA), can prevent translation of the message (Rothenberg *et al.*, 1989). Hybridisation of the complementary strand produces a duplex that is believed to be prone to digestion and this prevents translation of the mRNA (Paterson *et al.*, 1977). This technique, therefore, enables the selective removal of a species of mRNA from the cell, effectively inhibiting *de novo* synthesis of that protein. Using this approach, Tortora *et al.* (1990) employed a 21-mer oligodeoxynucleotide complementary to the $R_{II\beta}$ mRNA to determine its involvement in the growth of the leukaemic cell line HL-60. Exposure of cells to the $R_{II\beta}$ antisense sequence (15 μ M) for seven days had no effect on the rate of cellular proliferation. 8-Cl cAMP (10 μ M), in the absence of oligomer, inhibited proliferation by 70% and produced a morphological change. However, in the presence of oligomer, 8-Cl cAMP reduced the level of proliferation by only 20% and no change in morphology was evident. No detectable $R_{II\beta}$ protein was found in the presence of oligomer, and 8-Cl cAMP could not induce levels of $R_{II\beta}$. This work gave direct evidence that $R_{II\beta}$ regulatory subunit is required for the growth inhibitory effect and differentiation of HL-60 cells. Tortora *et al.* (1991) performed the same experiments using the $R_{I\alpha}$ antisense sequence on HL-60 cells. They demonstrated growth inhibition and monocyte differentiation with antisense $R_{I\alpha}$ (15 μ M) alone, a typical

response observed with 8-Cl cAMP. No further change in growth inhibition and cellular morphology was observed on addition of 8-Cl cAMP to cells treated with oligomer. Furthermore, cells treated with antisense RI_{α} had decreased levels of RI_{α} protein, concomitant with an increase in the RII_{β} product. Tortora *et al.* (1991) also showed that the growth inhibition and differentiation of HL-60 cells by phorbol esters was not affected by addition of antisense regulatory subunits. Their data suggest that the mechanism of action of 8-Cl cAMP with HL-60 cells is independent of that of phorbol esters.

cAMP Response Elements:

The RII_{β} subunit shares extensive homology with the cAMP receptor protein (CRP) or catabolite activator protein (CAP) in bacteria. CAP is a positive activator of the *lac* operon and is only active in the presence of cAMP. The activated CAP binds upstream of the *lac* promoter facilitating the formation of an active initiation complex (Ullman & Danchin, 1983). In light of accumulating data, RII_{β} is strongly implicated as a trans-acting factor.

CAP, and possibly RII_{β} , can directly modulate gene expression in the presence of cAMP through binding to specific DNA-binding sites, called cAMP response elements (CRE). There have been several cAMP-regulated genes identified in mammalian cells, that include chorionic gonadotropin (Silver *et al.*, 1987), somatostatin (Montminy *et al.*, 1987), and proenkephalin (Comb *et al.*, 1986). Within the first 150 base pairs of the 5'-flanking region of these gene promoters an eight base pair palindromic sequence was identified as the functional cAMP response element. A

chimeric construct of the DNA sequence containing the CRE sequence from the chorionic gonadotrophin was shown to confer cAMP inducibility to a heterologous promoter (Silver *et al.*, 1987). Furthermore, deletion studies of the 5'-flanking sequence and chimeric constructs with non-cAMP responsive genes have established this sequence as a cAMP responsive element (Short *et al.*, 1986; Montminy *et al.*, 1987; Comb *et al.*, 1986). Another cAMP-responsive element has been identified in the SV40 and human metallothionein II_A promoters (Imagawa *et al.*, 1987), termed Activator Protein 2 (AP-2). This element is not exclusively induced by cAMP, but also by phorbol esters. Addition of both agents have an additive effect on transcription of the gene (Mitchell *et al.*, 1987). Other genes identified with an AP-2 sequence include growth hormone (DeNoto *et al.*, 1981), prolactin (Cooke *et al.*, 1982), and plasminogen activator (Nagamine *et al.*, 1984).

Characterisation of these DNA sequences has enabled researchers to identify the nuclear binding proteins that associate to them. Montminy *et al.* (1987) purified a CRE binding protein (CREB) for the somatostatin gene which bound specifically to a 30 base pair sequence that contained the CRE. The protein had a molecular weight of 43 kDa and was phosphorylated *in-vitro* by the catalytic subunit of kinase A. The phosphorylation of this protein, however, did not alter the binding affinity for the CRE. Study of another CREB protein, which regulates the human chorionic gonadotrophin gene, also had no increase in binding affinity when phosphorylated (Delegeane *et al.*, 1987). Furthermore, the binding affinity of AP-2 protein, identified for the metallothionein II_A gene (Imagawa *et al.*, 1987), for its DNA element was not affected in the presence of cAMP. cAMP does not appear to affect the binding affinity of CREB and AP-2 proteins to their

respective regulatory elements in genes studied so far (Roesler *et al.*, 1988). Work by Nakagawa *et al.* (1988) provided strong experimental evidence suggesting the involvement of the kinase A catalytic subunit on gene transcription. Using the urokinase-type plasminogen activator gene, they demonstrated that in an *in-vitro* transcription assay the catalytic subunit stimulated transcription of this gene to a greater extent than cAMP. Grove *et al.* (1987) also indirectly implicated the importance of the catalytic subunit in transcription. These authors transfected a gene coding for a catalytic subunit inhibitor into cells which were also transfected with a cAMP-inducible gene. In this system, 90-95% inhibition of the induction of the cAMP-responsive gene by cAMP was reported. This evidence supports the role of the catalytic subunit as an intermediate in the effect of cAMP on gene transcription. Yamamoto *et al.* (1988) used a nuclear extract from PC12 cells to study the *in-vitro* transcription of a somatostatin-CAT fusion gene which contained an intact CRE. The nuclear extract was cleared of CRE binding protein using a CRE-affinity column, which allowed the authors to directly study a purified 43 kDa CREB protein (Montminy *et al.*, 1986). The technique of gel retardation, used to detect protein-DNA complexes, identified two bands that could be competed out by CRE sequences. Moreover, the higher molecular weight complex had a ten-fold greater affinity than the lower molecular weight, and was characterised and found to be a dimer of the CREB protein. This protein bound to the CGTCA sequence of the palindromic CRE sequence on each side of the DNA to form the dimer. Phosphorylation of the CREB proteins appeared to be important for the formation of the dimer, since treatment of CREB with alkaline phosphatase substantially reduced the levels of dimer formed. The CREB protein was also shown to be phosphorylated by kinase A and kinase C, but only kinase C appeared to

cause an increase in formation of the dimer. Moreover, kinase A treatment induced transcription by twenty-fold. Unfortunately, the effect of kinase C on transcription could not be confirmed because of technical problems. Nevertheless, these data suggest a possible dual regulation for CRE-dependent transcription between kinase A and kinase C. Cambier *et al.* (1987) showed that on addition of cAMP to B lymphocytes, kinase C translocated to the nucleus. This translocation appeared to be specific for cAMP and would support the suggestion of dual regulation by kinase A and kinase C. However, the translocation of kinase C in PC12 cells was not shown. Lamph *et al.* (1990) suggested that CREB could function as a negative regulator. The *c-jun* promoter contains a CRE sequence and the gene is induced by serum or forskolin. Addition of CREB represses the expression of *c-jun* on addition of serum to the cells and can be removed by phosphorylation of the CREB protein with kinase A or forskolin. This suggests that CREB protein could have a dual role in controlling the transcription activity of cAMP-responsive genes.

R11 β appears to be involved in the modulation of gene expression of cancer cells which results in growth inhibition and differentiation. Mednieks *et al.* (1989) demonstrated, with malignant cell lines treated with 8-Cl cAMP, that nuclear and cytoplasm extracts added to synthetic DNA containing CRE sequences had increased protein binding activity. Though 8-Cl cAMP results in the translocation of R11 β to the nucleus, these data do not prove that it is directly involved in DNA binding. However, R11 has been shown to bind to chromatin (Jungmann *et al.*, 1986; Sikorska *et al.*, 1988). Furthermore, it also binds to DNA *in-vitro*, as has been demonstrated by Constantinou *et al.* (1985), who suggested that the protein had properties of a topoisomerase. Direct binding of R11 to duplex

DNA has also been demonstrated by Wu & Wang (1989). These are the only evidence, to date, that suggests that RII can directly interact with DNA.

To investigate the role of RII β in regulation of CRE containing genes, Tortora & Cho-Chung (1990) stably transfected cAMP-unresponsive mutant pheochromocytoma cells (A126-1B2), which lack type II kinase A, with the RII β gene. These cells were then transfected with a somatostatin gene construct linked to a reporter gene. Expression of the RII β construct in these cells now conferred transcription activity of the somatostatin gene construct in the presence of cAMP. Therefore, RII β expression could confer cAMP responsiveness to a cell that was characterised as being deficient in type II kinase only. Expression of RI α could not confer cAMP induced gene expression of the reporter gene in these mutant cells. These data suggest a direct involvement of RII β in expression of genes with a CRE in a cAMP-dependent manner. It was therefore concluded that the RII β was essential for activation of the CRE-containing construct and that the regulatory subunit of type I was not. This evidence does not appear to support the previous view of researchers that the catalytic subunit is involved in gene transcription of this somatostatin construct, since these cells still have type I kinase A activity. It is possible that active type I kinase plus the presence of RII β is required for transcriptional activation. It is also possible that CRE-regulated transcription may involve the interaction of CREB and RII β with transcriptional activity regulated by kinase A and kinase C. CREB appears to have a dual function depending on its state of phosphorylation, acting as a repressor or activator. It does seem likely that the RII β subunit, which can also be phosphorylated by

kinase A (Taylor *et al.*, 1988), could have the potential for dual regulation of transcription in a similar manner as CREB. However, this has yet to be established.

Overview:

Site-selective analogues do appear to be a powerful tool for the control of malignant growth. There is accumulating evidence for the mechanism of action that clearly implicates a complex interaction of biochemical agents, including oestrogen receptors and kinases. Antisense work does suggest that $R11\beta$ and $R1\alpha$ are involved in the control of proliferation, but their role in cAMP-responsive gene induction is still not clear. Furthermore, studies of cAMP-responsive genes have been investigated in studies using addition of cAMP or forskolin, an activator of adenylyl cyclase, however, it would be interesting to know the effect cAMP analogues have on the expression of these genes and the effect these genes have on proliferation. 8-Cl cAMP has been shown to modulate oncogene, growth factor, differentiation markers, $R11\beta$, and $R1\alpha$ expression. These expression events have not been fully characterised in terms of when expression occurs on addition of 8-Cl cAMP, apart from $R11\beta$ which does appear to be an early event. A better understanding of the order genes are expressed on the addition of 8-Cl cAMP may give insight into its antiproliferative mechanism. It does seem likely that, since 8-Cl cAMP can replace cAMP for the activation of kinase A, it could also activate CREB and AP-2 proteins. It would, therefore, be important to understand the effect these cAMP-responsive gene products have on proliferation, and by doing so, verify if the effects of 8-Cl cAMP are indeed due to $R11\beta$,

CREB, AP-2 or combination of these three proteins. Understanding of the complex cascades that take place on addition of cAMP or cAMP analogues will help to identify the key components involved in control of proliferation and this, in turn, could lead to future development of other therapeutic agents.

INITIAL AIM OF THE THESIS:

The initial aim of this thesis is to investigate the mechanism of 8-Cl cAMP growth inhibition, especially in the hormone-responsive breast cancer line MCF-7. 8-Cl cAMP was reported by Katsaros *et al.* (1988) to antagonise the oestradiol growth stimulation of these cells, and this inhibition in growth was also associated with a decrease in the level of p21 and the R1 : R2 expression ratio. This investigation intends to elucidate how 8-Cl cAMP and oestradiol control the expression of p21, and determine if the concentration of p21 is involved in control of proliferation of these cells.

SECTION 1: **MATERIALS**

The following materials were obtained as indicated.

1.1 Radiochemicals:

From **Amersham International plc, U.K.;**

- Protein A, ^{125}I -labelled with Bolton and Hunter reagent (specific activity $>1.11 \text{ GBq/mg}$ total protein A)
- [methyl- ^3H] Thymidine containing 2% ethanol (specific activity 1.81 TBq/mmol)

From **ICN Radiochemicals, U.S.A.;**

- [^{32}P] 8-Azidoadenosine-3':5' cyclic monophosphate (specific activity $40\text{-}60 \text{ Ci/mmol}$);

1.2 Gel Electrophoresis:

Where ever possible Electrophoresis Purity Reagents were used.

From **Bio-Rad Laboratories Ltd., U.K.;**

- Acrylamide
- Ammonium Persulphate
- "Protein Assay"
- Piperazine Diacrylamide (PDA)
- N,N,N',N'-Tetramethylethylenediamine (TEMED)

From **Sigma Chemical Co. Ltd., U.K.;**

- (Coomassie)Brilliant Blue R
- Bromophenol Blue
- Glycine
- Lauryl Sulphate (Sodium Dodecyl Sulphate; SDS)
- 2-Mercaptoethanol
- Sucrose
- Tris[hydroxymethyl]aminomethane hydrochloride
- Tris[hydroxymethyl]aminomethane

1.3 Antibodies:

From **Sigma Chemical Co. Ltd., U.K.;**

- Anti-Actin (polyclonal)
- Anti-Rabbit IgG (whole Molecule)
- Anti-Rat IgG (whole Molecule)
- Anti-Tubulin (polyclonal)

Supplied by the **I.C.R.F. Antibody Production Unit;**

- BA17 (5 mg/ml total protein) monoclonal antibody against cytokeratin 19
- Y13-259 (600 µg/ml total protein) monoclonal antibody against p21^{ras}

From **Sera-lab, U.K.;**

- Monoclonal Anti-Tubulin

1. 4 General Chemicals:

These reagents are of Analar grade or equivalent.

From **FSA Laboratory Supplies;**

- Butan-1-ol
- Glycerol
- Hydrochloric Acid

From **Koch-Light Laboratories Ltd;**

- Isopropanol

From **May & Baker Ltd.;**

- Glacial Acetic Acid
- Methanol

From **BDH Ltd.;**

- Acetone
- Nonidet P 40 (NP40)
- Potassium Dihydrogen Orthophosphate (**Aristar™**)
- Sodium Chloride

- Sodium Hydroxide Pellets

From **Sigma Chemical Co. Ltd., U.K.;**

- Ammonium Sulfate
- Charcoal, activated
- Deoxycholic Acid, sodium salt
- Ethylenediaminetetra acetic acid (free acid)
- Magnesium Chloride, Hexahydrate
- 2[N-Morpholino]ethanesulfonic Acid
- Tetrabutylammonium Hydroxide

From **Rathburn Chemical Ltd.;**

- Methanol (HPLC grade)

From **MacKay and Lynn Ltd.;**

- Ethanol
- Methylated Spirits

1.5 Other Reagents:

From **Sigma Chemical Co. Ltd., U.K.;**

- Aprotinin
- 8-Bromoadenosine 3':5'-cyclic monophosphate
- Diethylaminoethyl Sephadex
- Dimethyl sulfoxide
- β -Estradiol
- Hydrocortisone
- Insulin (from Bovine Pancreas)
- Naphthol Blue Black
- Phosphodiesterase, 3':5'- cyclic nucleotide from bovine heart
- Retinoic acid (All Trans)
- Ribonuclease A (Bovine Pancreas)
- Sodium Selenite
- Sulfatase Type IV

- Transferrin (Human)

Kindly supplied by **Dr Y.S. Cho-Chung, National Institutes of Health, Bethesda, U.S.A.;**

- 8-chloro adenosine 3':5'-cyclic monophosphate

From **Pharmacia LKB.;**

- Dextran T70
- Sephacryl S-200 (Superfine);

From **Amersham International plc, U.K;**

- Rainbow™ Coloured Protein Molecular Weight Markers
(200-14.3 kDa)

From **Fluka;**

- Propidium Iodide

Kindly supplied by the **Imperial Chemical Industries, plc.;**

- Tamoxifen

From **Premier Brands, U.K. Ltd.;**

- Marvel (Dried Skimmed Milk)

1.6 Autoradiography:

From **Kodak Limited;**

- X-OMAT AR
- X-RAY FILM Developer
- X-RAY FILM Fixative

From **FUJI PHOTO CO. Ltd.;**

- Fuji X-RAY FILM

From **Genetic Research Instrumentation Ltd.;**

- X-ray Film Cassettes with Intensifying Screens

1.7 Cell Lines:

Supplied by the **Michigan Cancer Foundation, U.S.A;**

- Michigan Cancer Foundation 7 (MCF-7) epithelial breast cancer cell line

Supplied by the **Imperial Cancer Research Fund, London;**

- PEO1 epithelial ovarian cancer cell line
- HT-29 colorectal cancer cell line
- MDA-MB-231

Kindly supplied by **Dr R. G. Morris, Department of Pathology, Edinburgh University;**

- NIH/3T3 *v-ras*
- RFHO 6N1
- RFHO 208F

1.8 Tissue Culture:

From **Gibco Ltd.;**

- Dulbecco's Modified Eagle Medium with sodium pyruvate, with 1000mg/L glucose
- Dulbecco's Modified Eagle Medium without phenol red, with sodium pyruvate, with 1000mg/L glucose
- NewBorn Calf Serum
- RPMI with L-glutamine
- RPMI with L-glutamine, without phenol red
- Trypsin Solution 0.25% in Gibco solution A
- Penicillin (10000 IU/ml) - Streptomycin (10000 µg/ml) solution

From **Becton Dickson and Company;**

- Syringes and needles
- 25 cm², 75 cm², 175 cm² Tissue Culture Flasks

From **Northumbria Biologicals Ltd.;**

- Foetal Calf Serum

From **Sigma Chemical Co. Ltd., U.K.;**

- L-Glutamine

From **Flow Laboratories.;**

- Phenol Red 0.5% solution

From **OXOID Ltd.;**

- Phosphate Buffered Saline (Dulbecco 'A')

From **Costar;**

- 6 well tissue culture clusters

From **J.Bibby Scientific Products Ltd.;**

- 96 well flat bottom plates;

From **Sterilin;**

- 30 ml Universal containers and 7 ml Bijou bottles

1.9 Non-Chemicals:

From **Bio-Rad Laboratories Ltd.;**

- Trans-Blot Transfer Medium (0.45µm)
- Filter Paper Backing

From **Schleicher & Schuell;**

- Cellulosenitrat(E) 0.2µm

From **Whatman Limited;**

- Filter Paper no.1
- GF/B glass fibre paper
- Cellulose Nitrate 0.2 µm

From **Millipore Corporation;**

- Durapore Membrane Filter 0.45 µm
- GS 0.22 µM Filter Unit

From **Amicon;**

- Centricon™(10, 30, 50 &100 kDa MW cut off)
microconcentrators

SECTION 2:

METHODS

2.1 TISSUE CULTURE

2.1.1 Routine Maintenance of Cell Lines:

Culturing of cells was carried in a Class II hood and only sterile glass ware, plastics and solutions used.

All cell lines were routinely maintained in monolayer culture at 36.5 °C under an atmosphere of air/CO₂ (95%/5%). MCF-7, MDA-MB-231, HT-29, NIH/3T3 *v-ras*, RFHO 6N1 and RFHO 208F were cultured in 175 cm² and 75 cm² tissue culture flasks with Dulbecco's Modified Eagles Medium (DMEM) containing phenol red, supplemented with 2 mM glutamine and 10% (v/v) foetal calf serum (FCS) heat inactivated at 56 °C for 30 minutes. PEO1 and PEO4 cells were cultured in RPMI 1640 containing phenol red plus the other supplements as detailed above. All FCS used for routine passaging of cells and experiments was heat inactivated.

Every six or seven days confluent monolayers of cells were passaged into new flasks with fresh phenol red medium containing 10% FCS (v/v); this was done as follows:

- Old medium was discarded and the cell monolayer washed with a volume of PBS (a volume refers to enough liquid to cover the surface with an approximate depth of 0.5 cm).
- For MCF-7 cells a volume of 1 mM EDTA in PBS was put on the monolayer at room temperature, then removed after 5 minutes; this assisted the removal of the monolayer.
- A volume of 0.25% trypsin solution/versene (1 mM EDTA in PBS plus 0.5% (v/v) phenol red) [1:1]) was placed on the monolayer until it detached from the plastic.

- Fresh phenol red medium containing 10% FCS (v/v) (10 ml) was used to pipette the detached cells up and down against the side of the flask to break up cell clumps.
- A small volume of the cell suspension was then added to a new flask containing fresh medium containing supplements.
(Total volume: 25 cm²= 5 ml, 75 cm²= 20 ml, 75 cm²= 50 ml)

2.1.2 Mycoplasma Testing:

Once a month, medium (5 ml) was removed from confluent flasks of cells and tested for the presence of mycoplasma contamination (Barile, 1973) courtesy of Bill Christie, M.R.C. Human Genetics Unit, Edinburgh.

2.1.3 Banking Cells and Recovering:

Routinely, confluent flasks of cell were harvested by trypsinising of the monolayer of cells (section 2.1.1). The solution of cells was then aseptically transferred to a universal container and pelleted at 1000 g for 10 minutes. The liquid was removed and the pellet of cells resuspended in new born calf serum : dimethyl sulfoxide (1:1) with 1 ml for approximately every 10⁷ cells. The mixture was then aseptically transferred to a cryostat tube and placed in liquid nitrogen storage banks.

Cells were recovered by removing a cryostat tube and thawing the mixture. This mixture was then aseptically washed with PBS by repeated resuspension of the cell pellet with PBS and further centrifugation. This procedure was repeated three times before the cell pellet was finally resuspended in fresh medium containing supplements and transferred to a tissue culture flask (25 cm² or 75 cm²). Recovery of the cells would take 1-3 weeks, during that

period of time the cells were frequently washed with PBS and fresh medium and supplements added.

2.1.4 Plating Cells For Experiments:

A flask of cells were trypsinised from the plastic according to section 2.1.1. The cell suspension was transferred to a sterile universal container and syringed four times against the wall of the tube using a 21G1 $\frac{1}{2}$ needle. This produced a single cell suspension that could then be counted using a haemocytometer or Coulter counter™ (**Coulter Electronics**; see section 2.1.5 for settings) to determine the cell number per millilitre. An aliquot of cells was added to a volume of fresh medium with phenol red and 10% FCS (v/v), so that a volume for a given well would yield the correct number of cells when dispensed. This number would depend on the size of well used. For example, 6 well plates had 2 ml dispensed per well with approximately 5×10^4 cells per millilitre of medium to give 10^5 cells per well; 96 well plates received a volume of 250 μ l with 4×10^4 cells per millilitre giving a final cell count of 10^4 cells per well.

After a period of 18-24 hours to allow attachment of the cells to the plastic, the phenol red containing medium was aspirated off and replaced with fresh, phenol red free medium supplemented with 1% FCS (v/v) or HITS*. This growth medium was also supplemented with penicillin (100 I.U./ml) and streptomycin (100 μ g/ml).

*Experiments involving the use^{of} medium containing HITS had cells washed with HITS medium (section 2.2.1) only.

24 hours later, the medium was aspirated off and fresh phenol red free medium containing the appropriate supplements added to each well (see individual experiments). A set of wells were counted (see section 2.1.5) to determine the initial cell number per well before the addition of drug to each well. Every two or three days, old medium was aspirated off and fresh medium containing the appropriate supplements and drug added.

2.1.5 Counting Plated Cells:

At desired time intervals, medium was aspirated from the wells and a volume of 2.5 mM EDTA in PBS was added. PEO1 and PEO4 cells were not detached with this solution but with 1 mM EDTA in PBS/trypsin-versene solution (1:1). The solution was incubated on the cells for 15 to 30 minutes to allow the cells to detach from the plastic. The cells were broken into a single cell suspension using a 21G11₂ needle by syringing the cells against the wall of the well 4-5 times. The presence of a single cell suspension was checked under the inverted microscope (**Olympus**). 200 µl of this single cell suspension was added to a pot containing 9.8 ml of 0.9% (w/v) sodium chloride. Cell number was determined using a Coulter counter ZM with a 140 µm orifice and a sampling volume of 0.5 ml per count. The analyzer settings were such that the cell numbers determined by Coulter counter were similar to that of the haemocytometer. The reading from the counter was multiplied by 100 to convert the figure into cell number per millilitre.

$$\begin{aligned}\text{Cell No./ml} &= [\text{analyzer count}] \times [\text{pot volume/sample volume}] / \text{sampling volume} \\ &= [\text{analyzer count}] \times [10\text{ml}/200\mu\text{l}] / 0.5\text{ml} \\ &= [\text{analyzer count}] \times 100\end{aligned}$$

The cell number per well was calculated from cell number per millilitre multiplied by the volume of the solution used to detach the cells from the well. Three counts were determined for each pot (i.e. for each well).

2.1.6 Thymidine Uptake Experiments:

96 well plates were set up according to section 2.1.4. For example, MCF-7 cells were plated at approximately 10^4 cells per well. 72 hours after medium and drug were added, each well (containing 200 μ l) received 50 μ l of 0.37 MBq [methyl- 3 H] thymidine per millilitre of washing medium (medium used to wash off the phenol red medium after plating cells; see section 2.1.4). The plates were returned to the incubator for 6 hours. The radioactive waste medium was removed and the wells rinsed with ice cold PBS. Each well received 100 μ l of trypsin/versene solution for approximately 30 minutes to detach the cells. The amount of [methyl- 3 H] thymidine incorporated into DNA was determined using a 96 well tritium plate reader (**Canberra Packard**).



2.2 BUFFERS

All solutions were prepared using double distilled, deionised water at room temperature.

2.2.1 HITS Medium:

Hydrocortisone	10 nM
Insulin	5 µg/ml
Transferrin	10 µg/ml
Sodium Selenite	30 nM

These reagents were added to phenol red free media to give the above concentrations.

2.2.2 Buffer 10:

0.1M Sodium Chloride
0.5%(w/v) Sodium Deoxycholic acid
20 mM Tris.HCl (pH 7.4)
5 mM Magnesium Chloride
2 KIU/ml Aprotinin
1% (v/v) NP40

2.2.3 Buffer A:

20 mM Tris
0.25 M Sucrose
2 mM Magnesium Chloride
1 mM Calcium Chloride
10mM Potassium Chloride
16.26mM HCl

2.2.4 Electrophoresis Tank

Buffer:

25 mM Tris
0.2 M Glycine
3.5 mM SDS

2.2.5 Sample Buffer:

3% (w/v) SDS
15%(v/v) 2-Mercaptoethanol
30 mM Tris (pH 6.8)
30% (v/v) Glycerol
1% (v/v) Bromophenol Blue saturated solution

2.2.6 Transfer Buffer:

25 mM Tris

0.2 M Glycine

20% (v/v) Methanol

2.2.7 NTE-NP40: (pH 7.5)

50 mM Tris

0.15 M Sodium Chloride

2 mM EDTA

0.1% (v/v) NP40

2.3 PREPARATION OF CELL EXTRACTS

All procedures were performed at 0-4 °C.

2.3.1 Lysate:

Cell lysates were prepared using the detergent based Buffer 10 to enable the membrane associated proteins to be extracted into solution (Collins & Salton, 1979). Cells were collected from flasks, and cell number per ml determined (section 2.1.5). The cell suspension was centrifuged at 750 g for 10 minutes at 4 °C and the pellet resuspended with ice-cold PBS then re-centrifuged. A volume of Buffer 10 was added to the cell pellet (250 µl Buffer 10 per 10⁷ cells) and then sonicated using a micro ultrasonic cell disrupter (**KONTES**) for two 15 second periods, the sample was returned to ice between each period. The cell suspension was left on ice for 30 minutes and then centrifuged at 100 000 g for 30 minutes at 4 °C. The resulting supernatant was used as a cell lysate and the pellet kept for acid soluble protein extraction (see section 2.3.2).

2.3.2 Acid Soluble Protein Extraction:

A volume of 0.2 M hydrochloric acid (200 µl per 10⁷ cells) was added to the pellet that remained after preparation of the cell lysate (section 2.3.1). The pellet was vortexed vigorously until it dissolved. The suspension was centrifuged at 100 000 g for 30 minutes at 4 °C. The supernatant was then removed and neutralised using a solution of sodium hydroxide (final volume 250 µl per 10⁷ cells).

All extracts were used fresh or stored at -40 °C.

2.3.3 Cytosol:

The same procedure described for lysates (section 2.3.1) was used to prepare cytosols, except the non-detergent Buffer A was used instead of Buffer 10.

All extracts were used fresh or stored at -40 °C.

2.4 BRADFORD ASSAY (Bradford, 1976)

Protein concentration of cell extracts was determined using the Bradford Assay Kit (**Bio-Rad Laboratories**). The kit was used as a microtitre assay requiring only 10 µl of sample volume for protein determination. A known protein concentration of bovine serum albumin (supplied with kit) was used to construct a standard plot of nine concentrations (0 - 1.4 mg/ml) from which protein concentration of samples could be determined. Samples and standards were loaded into a 96 well plate and absorbance at 600 nm determined using a 96 well ELISA Plate Reader (**Bio-Rad Laboratories**). Construction of the calibration plot and determination of protein concentration was performed using the computer package AssayZap™ (**Biosoft, U.K.**).

2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

2.5.1. Preparation of Samples:

1/2 volume of sample buffer was added to a volume of cell extract containing 50-120 µg of protein. The mixture was heated to 95 °C for 3 minutes to denature the proteins. The mixture was allowed to cool before centrifuging in a micro-centrifuge for one

minute. Each sample was applied using a Hamilton syringe into a well in the stacking gel (see section 2.5.2). In addition to the samples, a volume of Rainbow™ markers (10 µl) was also prepared and applied to the gel as described.

2.5.2 Gel Set Up And Running:

SDS-PAGE (Laemmli, 1970) was performed using the Proteart™ II Slab Gel (**Bio-Rad Laboratories**) connected to an Electrophoresis Power Supply EPS 500/400 (**Pharmacia**). The resolving gels were constructed using 12% or 7.5% (w/v) Acrylamide : 0.32% or 0.2 % (w/v) PDA solution, respectively and 0.375 M Tris (pH 8.85) and 0.1% (w/v) SDS. This was mixed together in a conical flask, then 0.025% (w/v) ammonium persulphate and 0.025% (v/v) TEMED were added just prior to pouring the gel. To ensure that the surface of the resolving gel set horizontally a layer of water saturated butan-1-ol was placed on top after pouring the gel. The gel was allowed to polymerize for 30-60 minutes. The butanol was washed off the resolving gel with distilled water. A teflon well forming comb was inserted and the stacking gel solution containing 3.6% (w/v) Acrylamide : 0.1% (w/v) PDA solution, 0.125 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.025% (w/v) ammonium persulphate and 0.025% (v/v) TEMED was poured. After polymerization, the comb was removed and the wells rinsed out with tank buffer. The plate, containing the gel, was assembled and tank buffer added to the bottom and top reservoir. The wells were then loaded with prepared samples. The samples were run through the stacking gel at 70 mA and then 35 mA through the resolving gel. The run was completed when the Bromophenol blue dye reached the bottom of the resolving gel. The apparatus was then dismantled and the stacking gel removed from

the resolving gel.

The resolving gel could then be stained with Coomassie blue dye (see section 2.5.4) to visualise proteins or assembled in an electrotransfer tank to transfer protein to nitrocellulose (see section 2.5.5) or dried down (see section 2.5.3).

Non-denaturing gels were constructed without the use of SDS either in the gel or the sample buffer.

2.5.3 Gel Drying:

The resolving gel was soaked in a solution of 10% (v/v) glycerol, 10% (v/v) acetic acid and 40% (v/v) methanol for 45 minutes or overnight. The gel was then dried using a gel drier (**Pharmacia**) connected to a vacuum pump. The drying process took 2 hours.

2.5.4 Staining Gels:

Gels were immersed in a filtered solution of 40% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Bromophenol blue R overnight. Destaining was performed with 25% (v/v) methanol and 10% (v/v) acetic acid with the addition of diethylaminoethyl sephadex (0.2-0.5 g per 250 ml of destaining solution). Destaining took approximately 6 hours.

2.5.5 Electrotransfer:

The resolving gel was soaked in transfer buffer for 10 minutes. A sheet of nitrocellulose was soaked in distilled water for 10 minutes followed by a 10 minute soak in transfer buffer. The gel and nitrocellulose sheet were assembled into a cassette and immersed into the Trans-Blot™ cell

(**Bio-Rad Laboratories**) containing transfer buffer. Transfer was conducted at 4 °C, 60 volts with no current limit overnight. The apparatus was disassembled and the nitrocellulose sheet soaked in NTE NP40 buffer for 10 minutes at room temperature.

2.6 WESTERN BLOTTING (Burnette, 1981)

2.6.1 Western Blotting:

After electrotransfer (section 2.5.5) the nitrocellulose was transferred to NTE NP40 containing 3% (w/v) Marvel at 37 °C for 3 hours. The nitrocellulose was then incubated in fresh 3% (w/v) Marvel in NTE NP40 containing 10 µg/ml Y13-259 at 4 °C for 16 hours. After washing the nitrocellulose for two 10 minute periods at 4 °C with ice-cold NTE NP40 the second antibody, rabbit anti-rat IgG, was incubated on the blot in fresh 3% (w/v) Marvel in NTE NP40 at a concentration of 4 µg/ml for 90 minutes at 4 °C. The nitrocellulose blot was washed for a further two 10 minute periods at 4 °C before [¹²⁵I] Protein A in fresh 3% (w/v) Marvel in NTE NP40 (25 kBq/ml) was added to the nitrocellulose and incubated at 4 °C for 45 minutes. The blot was given a further two 10 minute washes of NTE NP40 at 4 °C and air dried. The nitrocellulose blot was wrapped in clingfilm and exposed to X-ray film (see section 2.8) to detect binding of the antibody to p21.

2.6.2 Other Antibodies Used For Western:

Primary antibodies included:- monoclonal anti-tubulin, polyclonal anti-tubulin, polyclonal anti-actin and monoclonal anti-cytokeratin 19.

The secondary antibodies used were IgG molecules *specific for* immunoglobulins of the host species used to produce the primary antibody.

2.6.3 Staining Nitrocellulose Blots:

A nitrocellulose blot was immersed in a solution of 0.1% (w/v) Naphthol blue black, 25% (v/v) isopropanol and 10% (v/v) acetic acid for 30 seconds. The blot was destained with two volumes of 25% (v/v) isopropanol and 10% (v/v) acetic acid for one hour each wash and then allowed to air dry.

2.7 PHOTOAFFINITY LABELLING (Pomerantz *et al.*, 1975)

4×10^{-7} M [^{32}P] 8-Azidoadenosine-3':5' cyclic monophosphate in an solution containing 0.27 M 2[N-morpholino]ethanesulfonic acid and 53 mM magnesium chloride was added to 50-150 μg cytosol protein (section 2.3.3). The mixture was incubated at 20 °C for 60 minutes in the dark. The reaction mixture was then irradiated for 15 minutes at 254 nm using a Universal UV Lampe (**Camlab**). The mixture was then prepared for loading (section 2.5.1) onto a 12% SDS-PAGE gel (section 2.5.2). The gel was dried (section 2.5.3) and then exposed to X-ray film (section 2.8).

2.8 AUTORADIOGRAPHY

2.8.1 Autoradiography: (Sambrook *et al.*, 1989)

Two types of X-ray film were used, X-OMAT, which was the most sensitive, and FUJI. Both types of film were preflashed first before placing the film next to the gel or nitrocellulose.

Preflashing X-ray film using a flash unit covered in safety light filter (**Kodak, Wratten 22A**) and filter paper (to reduced the brightness of light flashed at film) sensitises the silver halide crystals making it more sensitive to the radioactivity

(Laskey & Mills, 1977). The required distance between the film and the flash unit was determined by varying the distance and/or changing the amount of filter paper covering the flash unit. Preflashed and unexposed (unflashed) film was then developed and pieces placed into a dual beam spectrophotometer at 545 nm (**Phillips**). The correct distance was determined when the absorbance reading was between 0.1-0.15.

Autoradiography was carried out at -70 °C for varying lengths of time until an adequate intensity of staining developed to enable scanning.

Example of exposure times:-

[¹²⁵I] Protein A = 2-8 hours

[³²P] 8-Azido cAMP = 2-24 hours

X-ray film was developed for 3 minutes in a 25% (v/v) developer solution and then for 1 minute in a 25% (v/v) fix solution. The X-ray film was washed in water for 10 minutes before being air dried.

2.8.2 Autoradiograph Scanning:

Scanning was performed on a non-commercial, pixel density analyzer constructed by Daryl K. Green Medical Research Council, Edinburgh. Staining densities were computer generated and the area under the peak determined to give a quantitative figure in arbitrary units.

2.9 CHARCOAL STRIPPING SERUM (Lippman *et al.*, 1976)

2.5 g of charcoal was added to 100 ml of distilled water and mixed for 5 minutes. The charcoal was allowed to settle and the fines floating on the surface were removed using a dry piece of filter paper. This mixing and removing was repeated until all the fines were removed. 0.25 g of dextran was added and mixed followed by addition of Tris to bring the volume to a final concentration of 0.01 M. The pH of the solution was adjusted to 7.4 using HCl and then centrifuged at 25 000 g for 10 minutes to produce charcoal/dextran pellets. 500 ml of FCS was incubated with a 1000 units of sulphatase for 2 hours at 37 °C. This was then added to the charcoal/dextran pellet. The mixture was incubated at 56 °C for 30 minutes and centrifuged at 25 000 g for 10 minutes. The FCS was carefully removed from the pellet and then added to another charcoal/dextran pellet prepared as detailed above. This was then mixed for 16 hours at 4 °C. The mixture was then centrifuged again and the double stripped FCS carefully removed from the pellet. The FCS serum was sterilised through a 0.22 µm filter units.

The efficiency of the protocol for stripping out oestradiol was checked by adding a small quantity of [³H] oestradiol before treating the serum. After stripping, a measure of the activity remaining in the serum was measured using a scintillation counter and related to the initial activity before stripping.

97% of the endogenous oestradiol was removed.

2.10 DNA ANALYSIS BY FLOW CYTOMETRY

(Danova *et al.*, 1990)

Cells were harvested and counted according to protocol (section 2.1.5). Aliquots containing approximately 10^6 cells per tube were collected by centrifuging at 1000 g, 4 °C for 10 minutes. The cells were washed by resuspending pellets in PBS and then re-centrifuged. The cell pellets were resuspended in 70% ethanol (v/v) and placed at 4 °C for 30 minutes. Pellets were collected by centrifuging and then washed once in PBS. 100 µl of 5 mg RNAase /ml of PBS and 100 µl of 100 µg/ml of propidium iodide dissolved in PBS were added to each pellet and incubated in the dark for 15 minutes at room temperature. The sample was analyzed on a Flow Cytometer (**FACSCAN; Becton Dickson**); DNA staining was detected at 564 - 607 nm wavelength.

2.11 FILTRATION TECHNIQUES

2.11.1 Concentrating Serum:

Serum was concentrated using different microconcentrators containing a specific molecular weight cut off filter for separating proteins of different molecular weights.

A volume of PBS was first spun through the column to remove the preservatives from the filter. The PBS was discarded and a sample of serum was placed on top of the filter and centrifuged in a fixed angle head at 4 °C until the sample volume stopped reducing.

The centrifugal force depended on the filter being used; Centricon 10 kDa and 30 kDa filters were centrifuged at 6000 g, Centricon 100 kDa at 1000 g.

2.11.2 Gel Filtration:

Sephacryl S-200 was mixed with PBS and poured into a glass column (10 cm x 7 mm diameter, 11 cm x 18 mm diameter). Three volumes of PBS were used to wash through the column. A concentrated sample of serum (section 2.11.1) was then applied to the top of the column and eluted using PBS. Fractions were collected every 10 minutes and the presence of protein detected by measuring the absorbance of a small sample of the fraction with a spectrophotometer at 280 nm.

2.11.3 Sample and Buffer Preparation for High Pressure Liquid Chromatography (HPLC):

The buffer was a solution of 8 mM potassium dihydrogen orthophosphate and 4 mM tetrabutylammonium hydroxide. The buffer was filtered through 0.2 μ m cellulose nitrate filter and HPLC grade methanol was filtered through 0.45 μ m Durapore filter.

Each sample was centrifuged through a Centricon 10 kDa filter at 6000 g until all the liquid had passed through the filter. The filtrate was then used for HPLC analysis (section 2.11.4).

2.11.4 High Pressure Liquid Chromatography (HPLC)

Analysis: (Van Lookeren Campagne *et al.*, 1991)

Analysis of 8-Cl cAMP and metabolites was studied using a reverse-phase μ Bondapak™ C₁₈ column (3.9 mm internal diameter x 30 cm) with a particle size of 10 μ m. The mobile phase was a 70% : 30% mixture of buffer and methanol with a flow rate of 1.4 ml/minute. Sample was mixed 1:10 (v/v) with mobile phase and a 50 μ l volume injected using a WISP autoinjector (**Waters**

Associates). Detection of the column elution was monitored at 254 nm using an Absorbance Detector (**Waters Associates**). Analysis of the chromatogram and integration of the peaks was carried out using the MAXIMA computer program (**Waters Associates**). Alternatively, the C₁₈ column was attached to a HP1090 Liquid Chromatography (**Hewlett Packard**) and samples injected using the same conditions as stated above.

SECTION 3:

RESULTS

3.1 PARTIAL CHARACTERISATION OF THE MCF-7 CELL LINE

3.1.1 Introduction

MCF-7 cells originated from the pleural effusion of a patient with metastatic breast cancer (Soule *et al.*, 1973). This established cell line contained oestradiol receptors and on addition of physiological levels of oestradiol resulted in an increase in rate of proliferation (Lippman *et al.*, 1976; Lippman, 1980). These cells have been used widely as a model system for hormone-responsive breast cancer. For example, MCF-7 cells have been used to study the effects of agents, such as tamoxifen and retinoic acid, as potential drugs for the treatment of hormone-responsive breast cancer (Katzenellenbogen *et al.*, 1987; Sonnenschein *et al.*, 1985; Fontana *et al.*, 1987; Lacroix & Lippman, 1980).

3.1.2 Hormone-Responsiveness:

MCF-7 cells were obtained direct from the Michigan Cancer Foundation at passage number 145. These cells were continually passaged weekly in phenol red DMEM containing 10% FCS (v/v) (see section 2.1.1). All experiments were carried out using phenol red free DMEM; phenol red has been identified as having an oestrogenic effect (Berthois *et al.*, 1986).

Pilot growth experiments were carried out to determine a suitable concentration of serum required in the culture medium to demonstrate an oestrogenic response with these MCF-7 cells. Figure 1(a) shows a representative growth experiment for the cells cultured in DMEM containing 1% charcoal-stripped FCS (v/v). Cells were plated with a density of approximately 10^5 cells per

well in a 6 well plate and incubated with this medium for approximately seven days (see section 2.1.4 for protocol). The population doubling time was determined from the computer calculated equation based on an exponential line fit for all time points (Cricket Graph computer program v1.3, **Apple MacIntosh**). Cells grew with a doubling time of 103 hours \pm 26 (n=6; errors represent standard deviation).

Figure 1(a) also shows the response of these MCF-7 cells to exogenous oestradiol (10^{-8} M). The rate of cellular proliferation was increased over the basal growth rate (control) by approximately two fold. This growth stimulation gave a doubling time of 44 hours \pm 2 (n=5); addition of 10^{-9} M oestradiol gave 45 hours \pm 2 (n=3). Two concentrations of tamoxifen (0.5 μ M and 1 μ M) added to MCF-7 cells with oestradiol (10^{-8} M) appeared to inhibit the rate of proliferation (Figure 1(a)). Furthermore, the higher concentration of tamoxifen had a greater degree of growth inhibition. Addition of tamoxifen to MCF-7 cells cultured in the absence of oestradiol also appeared to inhibit growth, but to a lesser degree in the presence of oestradiol, and was not concentration dependent. Addition of retinoic acid (1 μ M) to these cells showed a similar growth inhibitory effect as for tamoxifen (Figure 1(b)).

These data demonstrate that for the MCF-7 cells, cultured in DMEM containing 1% charcoal-stripped FCS (v/v), have a typical growth response to oestrogen, tamoxifen and retinoic acid as described in the literature.

3.1.3 Oestrogen Receptor Content:

The oestrogen receptor content of these MCF-7 cells was determined from four samples passaged between 147 - 192, cultured in phenol red DMEM containing 10% FCS (v/v). The oestrogen receptor assay was kindly performed by Dr R. A. Hawkins' Laboratory (Department of Surgery , Royal Infirmary, Edinburgh) using an ER/EIA monoclonal kit (supplied by ABBOTT Laboratories), which gave an average oestradiol receptor content of $138 \text{ fmol} \pm 28$ receptor sites / mg soluble protein (n=4; errors represent standard deviation).

The presence of oestrogen receptors, the growth stimulation by oestradiol and the growth antagonism by tamoxifen and retinoic acid characterises this cell line as hormone responsive in nature.

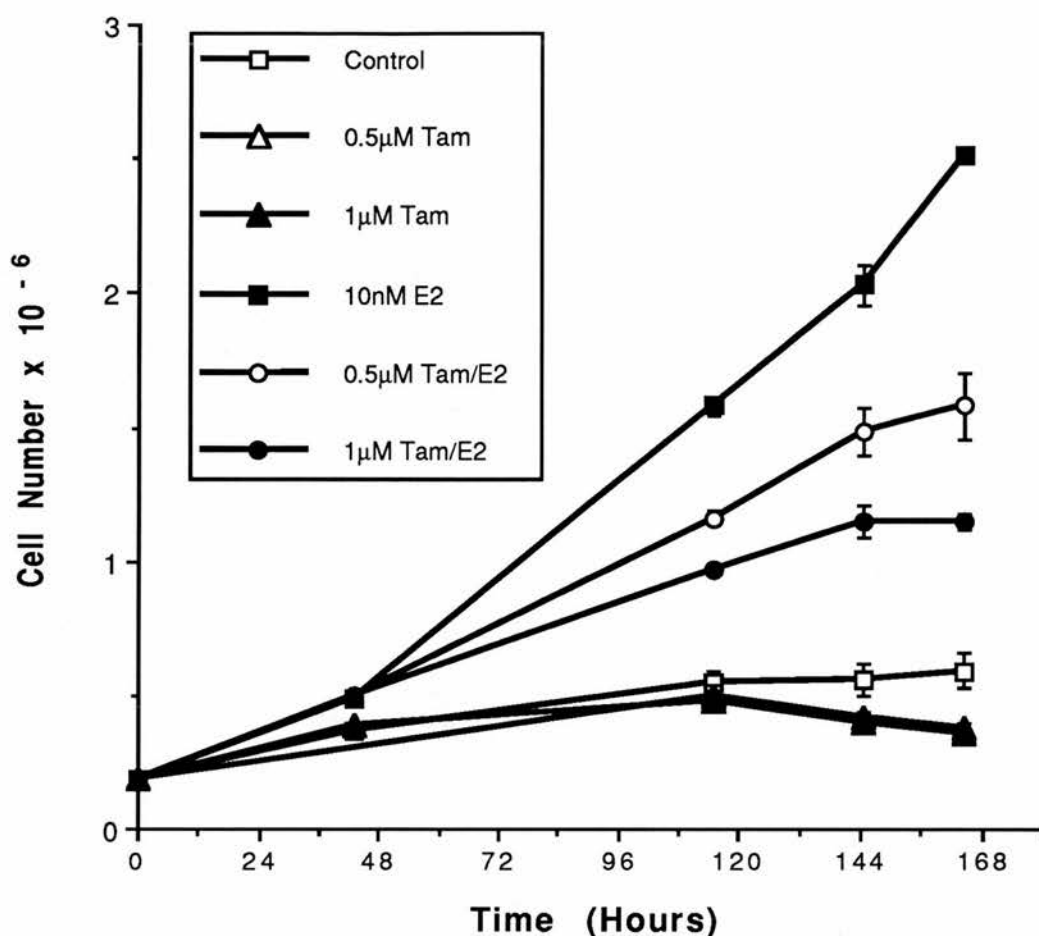


FIGURE 1(a):

The effect of oestradiol (E2), tamoxifen (Tam) and combinations of oestradiol and tamoxifen (Tam/E2) on growth of MCF-7 cells cultured in DMEM containing 1% charcoal-stripped FCS (v/v). MCF-7 cells were plated in 6 well plates and counted according to sections 2.14 and 2.1.5.

Data are the means of triplicate values \pm standard deviation of a representative experiment.

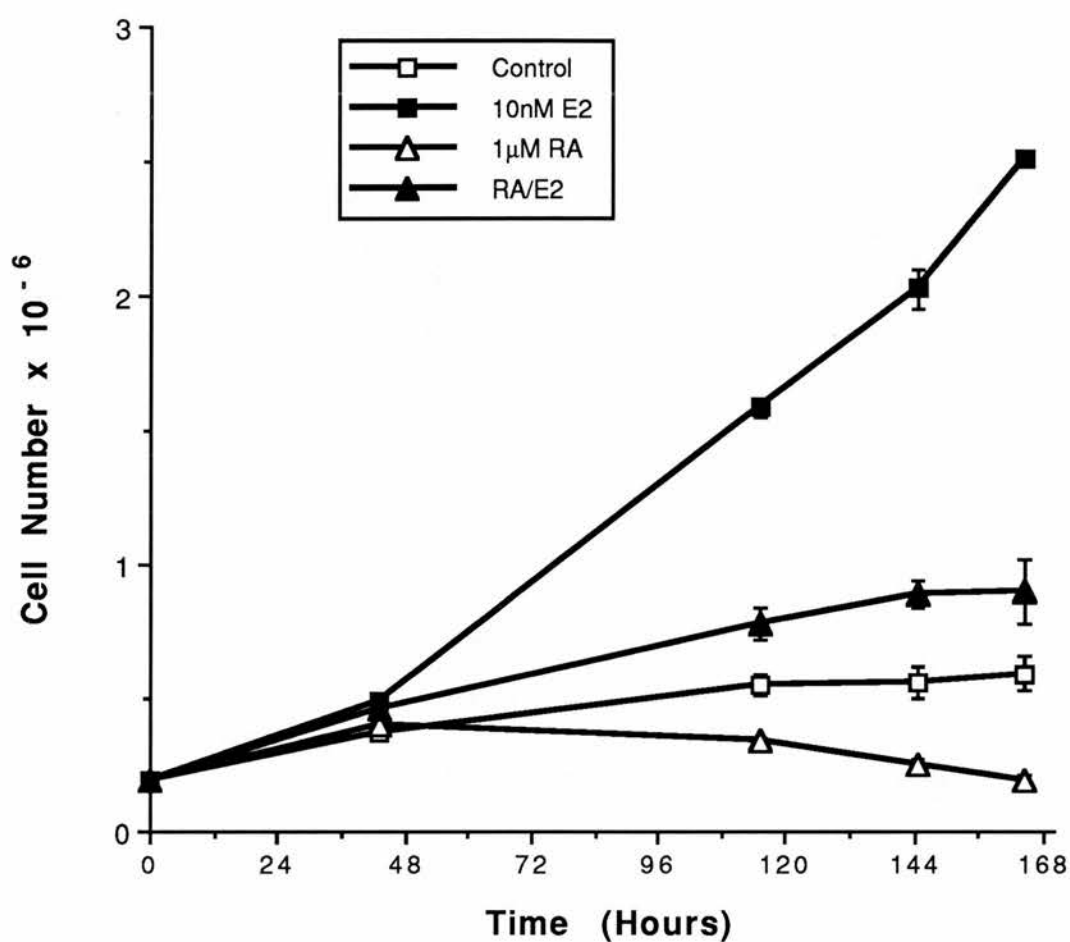


FIGURE 1(b):

The effect of retinoic acid (RA), oestradiol and combination of retinoic acid and oestradiol (RA/E2) on growth of MCF-7 cells cultured in DMEM containing 1% charcoal-stripped FCS (v/v). MCF-7 cells were plated in 6 well plates and counted according to sections 2.14 and 2.1.5.

Data are the means of triplicate values \pm standard deviation of a representative experiment.

3.2 THE EFFECT OF 8-Cl cAMP ON GROWTH OF MCF-7 CELLS IN CULTURE

3.2.1 Introduction

The reported concentration of 8-Cl cAMP that gives 50% growth inhibition (IC_{50}) after 5 days in MCF-7 cells is $1\mu M$ (Katsaros *et al.*, 1988). These MCF-7 cells were stimulated by a two fold increase with oestradiol (10 nM) cultured in medium containing 6.5% charcoal-stripped FCS (v/v). The authors demonstrated that this growth stimulation could be inhibited by 8-Cl cAMP ($10\mu M$). Furthermore, 67% growth inhibition was also observed after 3 days by $50\mu M$ 8-Cl cAMP with MCF-7 cells cultured in medium containing 10% unstripped FCS (Katsaros *et al.*, 1987).

3.2.2 Antiproliferative Effects Of 8-Cl cAMP:

MCF-7 cells were set up in 6 well plates according to section 2.1.4 and cultured in DMEM containing 1% charcoal-stripped FCS (v/v) with oestradiol (10 nM) and 8-Cl cAMP ($10\mu M$). Cell numbers were determined for each plate (section 2.1.5) at time points indicated on Figure 2.

The oestradiol growth stimulation appeared to be unaffected by 8-Cl cAMP (Figure 2). Moreover, 8-Cl cAMP ($10\mu M$) did not have an effect on the basal growth rate of MCF-7 cells (control). These data suggest that in these culture conditions MCF-7 cells are not growth inhibited by $10\mu M$ 8-Cl cAMP.

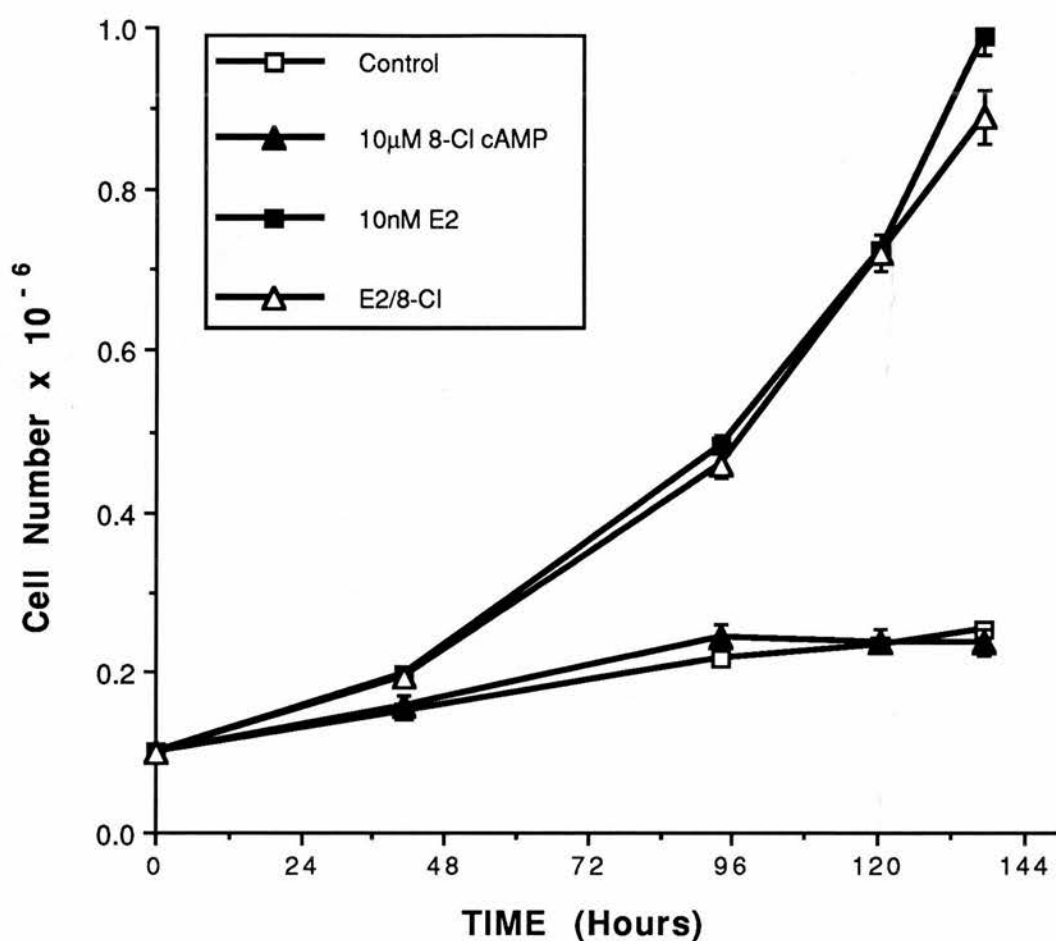


FIGURE 2:

The effect of oestradiol (**E2**), 8-Cl cAMP and combination of oestradiol and 8-Cl cAMP (**E2/8-Cl**) on growth of MCF-7 cells cultured in DMEM containing 1% charcoal-stripped FCS (v/v).

Data are the means of triplicate values \pm standard deviation of a representative experiment.

3.3 THE EFFECT OF SERUM ON INHIBITION OF MCF-7 CELLS BY 8-Cl cAMP WITH MCF-7 CELLS

3.3.1 Introduction:

To determine if the absence of an antiproliferative effect on MCF-7 cells by 8-Cl cAMP (10 μ M) (see section 3.2) was due to the low serum concentration and/or charcoal-stripping of serum, MCF-7 cells were cultured in medium containing different concentrations of serum.

3.3.2 Serum-Dependent 8-Cl cAMP Growth Inhibition:

Growth experiments with MCF-7 cells were set up according to section 2.1.4 with approximately 10⁵ cells/ well of a six well plate. Cells were cultured in media containing different concentrations of charcoal-stripped or unstripped FCS in the presence or absence of oestradiol (10 nM) and 8-Cl cAMP (10 μ M).

Figure 3 shows a bar chart with each bar representing the percentage of treated cell number relative to the control cell number (i.e. in the absence of drug; 100%) after a 120 hour incubation. Addition of 8-Cl cAMP (10 μ M) to MCF-7 cells cultured in DMEM containing 1% unstripped or charcoal-stripped FCS (v/v) did not appear to have an effect on growth. However, growth inhibition by 8-Cl cAMP was evident with MCF-7 cells cultured in unstripped or charcoal-stripped 10% FCS (v/v). Furthermore, growth inhibition was also present with 5% unstripped FCS (v/v), but the degree of inhibition was less than for 10% FCS (v/v). Growth stimulation by oestradiol had a more pronounced effect with MCF-7 cells cultured in lower concentrations of serum; maximum stimulation with 1% charcoal-stripped FCS (v/v).

However, the results for growth of MCF-7 cells cultured in different serum concentrations with oestradiol and 8-Cl cAMP appeared similar for cells in the absence of oestradiol. In other words, the growth inhibition of MCF-7 cells appeared to be dependent on the concentration of serum in the medium, irrespective of the presence of oestradiol.

3.3.3 Non-Antagonistic Effect of 8-Cl cAMP:

The numerical data from Figure 3 are presented in Table 1. Comparison is made between the degree of oestrogenic growth stimulation of MCF-7 cells cultured in the absence and the presence of 8-Cl cAMP. From these data it appears that 8-Cl cAMP had no effect on the oestrogenic stimulation of MCF-7 cells cultured with different concentrations of serum. This suggests that this cAMP analogue does not antagonise the oestrogenic effect as described by Katsaros *et al.* (1988).

I don't
agree
that
Table 1
shows
this:
certainly not
unambiguously

(cf Row 3 vs row 2
data)

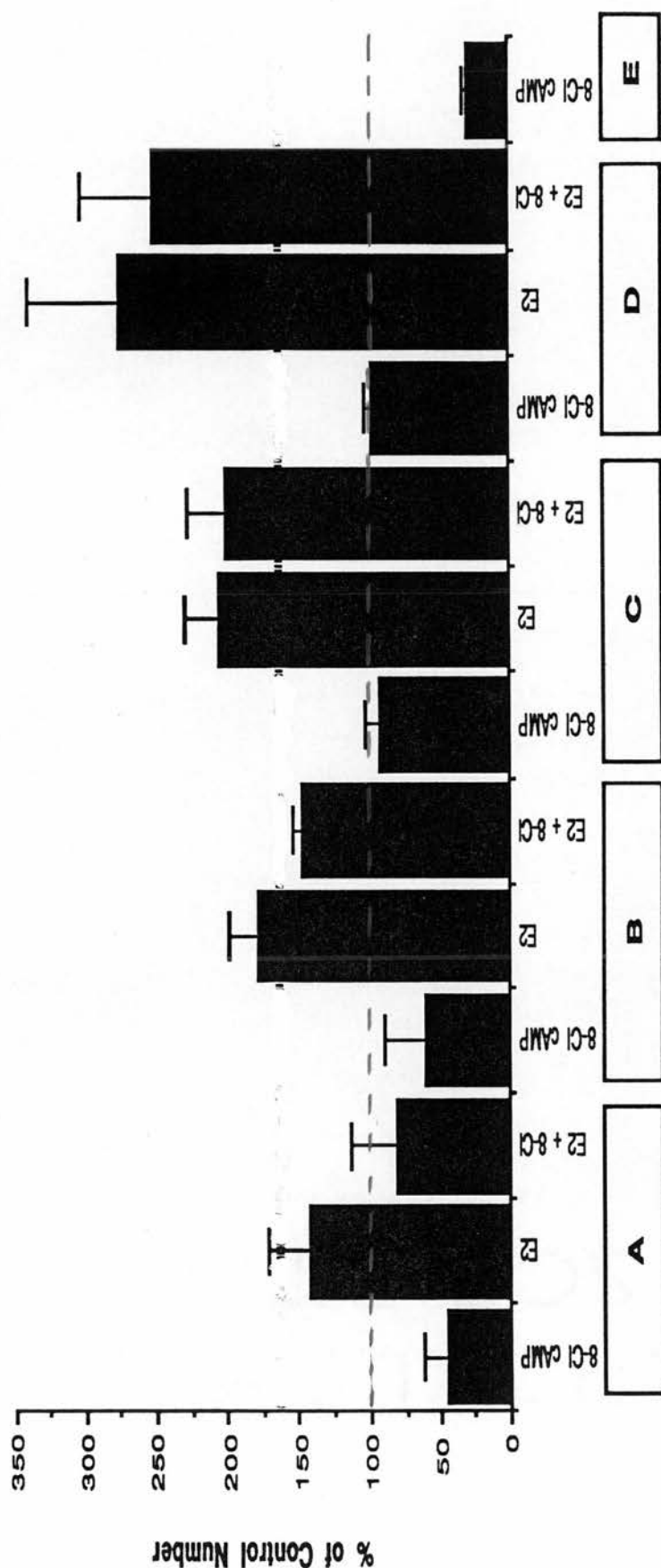


FIGURE 3:
 The effect of 8-Cl cAMP (10 μ M), oestradiol (E2; 10 nM) and combination of both (E2 + 8-Cl) on growth of MCF-7 cells cultured for 120 hours in DMEM containing-
A: 10% FCS, **B:** 5% FCS, **C:** 1% FCS, **D:** 1% charcoal-stripped FCS and **E:** 10% charcoal stripped FCS (v/v).
 Cell numbers in each experiment are expressed as a percentage of the control culture to which neither 8-Cl cAMP nor oestradiol was added. Each bar represents the mean of at least three experiments \pm standard deviation.

% FCS (v/v)	10%	5%	1%
1. 8-Cl cAMP (10 μ M)	45%	60%	92%
2. Oestradiol (E2; 10nM)	142%	179%	205%
3. E2 + 8-Cl cAMP	81%	148%	201%
4. Fold stimulation in growth by E2	1.42	1.79	2.05
5. Fold stimulation in growth by E2 in the presence of 8-Cl cAMP	1.80	2.47	2.18

Table 1:

Effect of oestradiol (E2; 10 nM), 8-Cl cAMP (10 μ M), and combination of both drugs on the growth of MCF-7 cells cultured for 120 hours in different %FCS (v/v) containing DMEM (data taken from Figure 5, see legend).

Row 1,2 & 3 data are expressed as a percentage of drug treated cell number against control cell number (absence of drug).

Row 4 gives the ratio of cell number for oestradiol treated cells against control cell number (fold stimulation by oestradiol).

Row 5 gives the ratio of cell number for cells treated with oestradiol and 8-Cl cAMP against 8-Cl cAMP treated cell numbers (fold stimulation by oestradiol in the presence of 8-Cl cAMP).

3.4 EXTRACTION OF PROTEIN FROM CELLS

3.4 Introduction:

p21 is bound to the plasma membrane and can be extracted with a double detergent buffer containing Nonidet P40 and sodium deoxycholate (Debortoli *et al.*, 1985, Katsaros *et al.*, 1987, Tagliaferri *et al.*, 1988). These groups normalised their samples to a fixed amount of protein and Western blotted with the monoclonal Y13-259 to detect p21. They scanned the intensity of p21 staining on X-ray film by densitometry and expressed the arbitrary units normalised to the amount of protein for each sample. Using this approach Katsaros *et al.* (1988) and Tagliaferri *et al.* (1988) reported a concomitant decrease in the level of p21 with growth inhibition of MCF-7 cells by 8-Cl cAMP.

Before this approach can be adopted to study p21 with the cells used in this study, it is necessary to demonstrate that protein can be extracted consistently for different samples.

3.4.2 Protein Content of Different Cells:

MCF-7 pellets containing different amounts of cells were prepared into lysates (see section 2.3.1) using Buffer 10, the double detergent buffer used by the above mentioned authors. The protein concentration for each lysate was determined using the Bradford Assay (section 2.4).

The original method described by Debortoli *et al.* (1985) disrupted cells using fine syringe needles and cleared the lysate with a 750 g centrifugation. Protein determination of samples prepared in this way did not produce a consistent concentration from lysates prepared from pellets containing the same cell

number. However, use of a sonicator instead of needles, and a 100 000 g centrifugation to clear the lysate of debris, produced a linear extraction of protein for known cell numbers as shown in Figure 4. The line through the points does not intersect zero because of the protein aprotinin, a proteinase inhibitor, added to Buffer 10. Preparation of lysates from RFHO 206F and RFHO 6N1 cell pellets also produced a similar plot. From the equation of these lines the total protein per cell was calculated.

<u>Cell Line</u>	<u>protein per cell</u>
MCF-7	= 218 pg
RFH06N1	= 98 pg
RFHO208F	= 218 pg

Equations of the line were calculated from plots using the Cricket Graph computer package, such as Figure 4, and used to determine the protein content per cell. Each value is the average of at least two plots.

These data demonstrate that a linear relationship exists between cell number and concentration of extract protein from a lysate. A fixed amount of protein can therefore be related to a known number of cells. However, the amount of extractable protein may differ for other cell lines. This would make comparison of different cell lines normalised to a fixed amount of protein difficult.

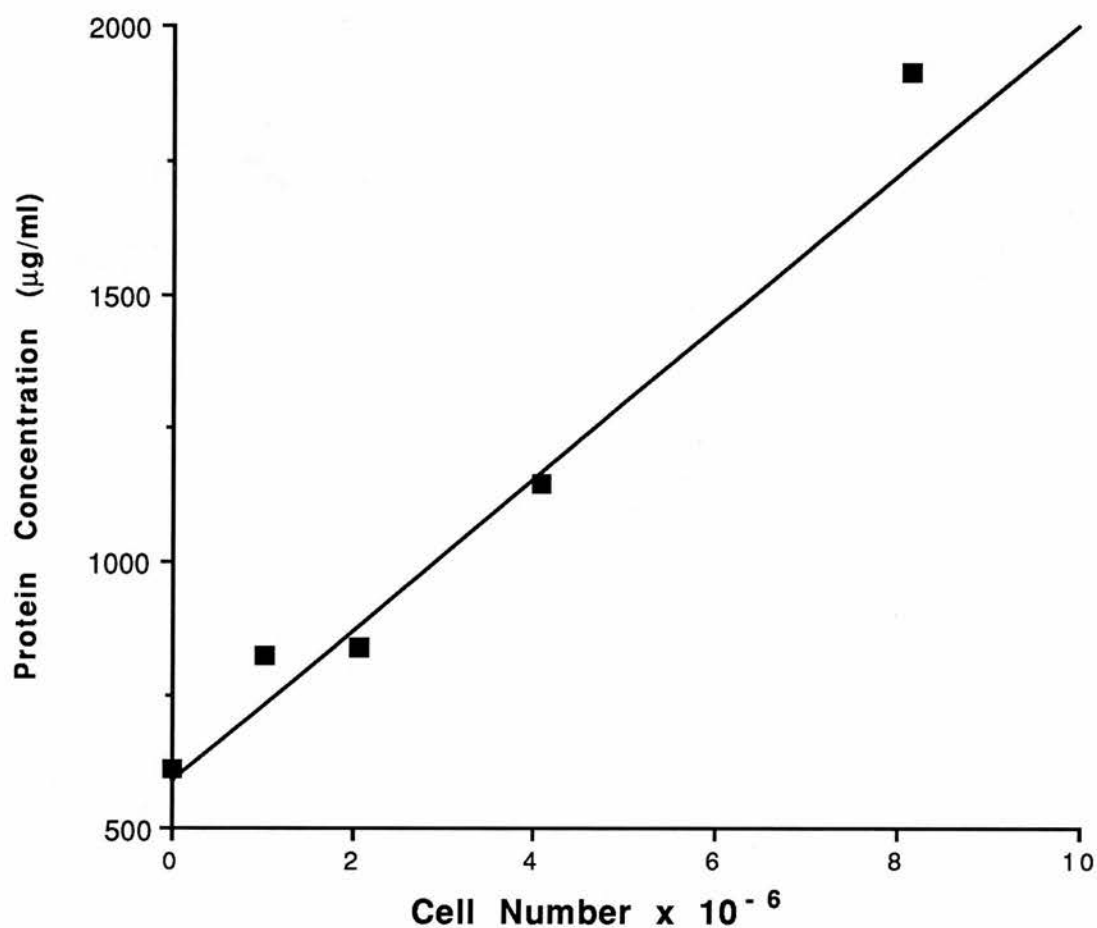


FIGURE 4:

Protein concentration of cell lysate prepared from MCF-7 cells, determined by the Bradford Assay
Data are the means of duplicates.

3.5 QUANTITATIVE ANALYSIS OF p21 BY WESTERN BLOTTING

3.5.1 Introduction:

Western blotting involves the detection of protein using specific antibodies (Burnette, 1981). Protein is fixed onto a membrane support, usually transferred from a PAGE gel, which can then be treated with antibody followed by an appropriate detection system such as radiolabel or enzymatic probes.

Y13-259 is a rat monoclonal antibody specific for a single epitope of p21. However, it does not discriminate between the different *ras* gene products (Shih *et al.*, 1979; Furth *et al.*, 1987). A double antibody technique was employed, this involved cross-linking Y13-259 bound to p21 with an anti-rat IgG. Detection and subsequent quantification of p21 was accomplished by the addition of radiolabelled ¹²⁵I-protein A which has a high affinity for IgG (Debortoli *et al.*, 1985) (section 2.6). The quantity of p21 fixed to the blot was determined by autoradiography (section 2.8). Radioactivity exposed to X-ray film activates the local silver halide crystals in the film. Development of the film results in a black stain and the intensity of the stain used as an indication of the amount of radioactivity present (Sambrook *et al.*, 1989).

3.5.2 Quantitative Analysis Of p21:

To determine the relationship between the intensity of the autoradiographic signal and the quantity of p21, a single lysate prepared from MCF-7 cells (section 2.3.1) was used to load the wells of a 12% SDS-PAGE gel (see sections 2.5.1 and 2.5.2) with decreasing amounts of protein from 120 to 10 µg. The resolved proteins were transferred from the gel to nitrocellulose

membrane (section 2.5.5). The membrane was Western blotted (section 2.6.1) and exposed to preflashed or unflashed X-ray film (see section 2.8.1) for a period of 3 hours before developing and scanning (section 2.8.2).

The staining of p21 by Y13-259 was very specific and produced very little background staining (Plate 1). Staining for p21 with 20 μg of lysate is just visible to the eye. To allow direct comparison of the scanning data for the two pieces of X-ray film, the data for each film was divided by the value for the 100 μg sample (i.e. 100 μg = 1 arbitrary unit) (Figure 5). A linear relationship appears to exist for the scanning data with respect to the concentration of protein present on the blot. The 10 μg lysate appears to be at the limit of detection by this technique for p21 detection, since the lines does not intersect the origin. The linear regression coefficient was improved when the X-ray film was preflashed before the blot was exposed compared to unflashed film; 0.975 and 0.923, respectively. These observations support the data of Laskey and Mills (1975 & 1979). They described how preflashing X-ray film before exposure to radioactivity could improve the linear response of the film.

The actual quantity of p21 can not be determined from this method because the concentration of p21 in the original lysate was not known. The concentration of p21 could be determined using a known concentration of pure p21 to calibrate the staining on X-ray film. Since pure p21 was not available absolute values could not be determined; however results can be expressed in units relative to a specific standard.

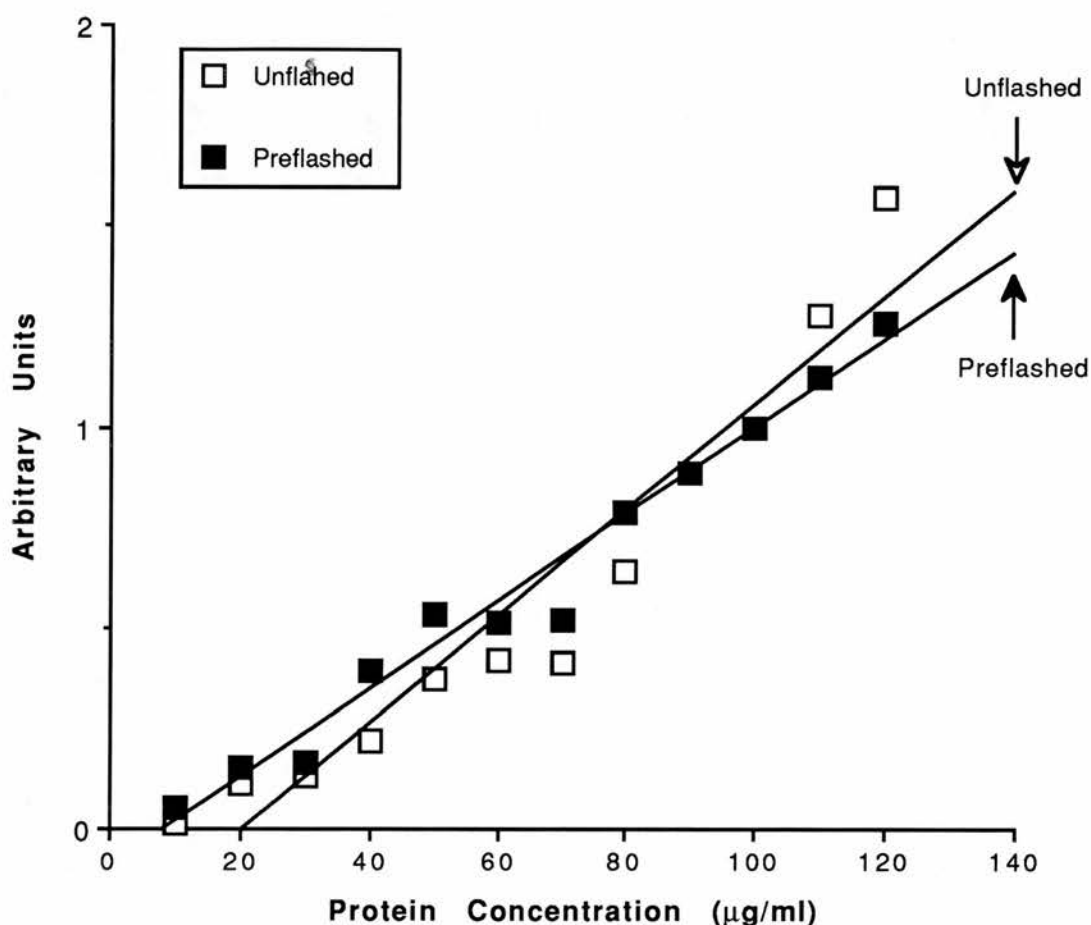


FIGURE 5:

Measurement of p21 by Western blotting with anti-p21 monoclonal (Y13-259) of varying protein concentrations of an MCF-7 cell lysate.

The blot was exposed to preflashed and unflashed X-ray film for 3 hours. Each point represents the autoradiographic signal, quantified by scanning and divided by the value for 100 µg/ml (arbitrary unit= 1).

Linear Regression Coefficient for	UNFLASHED	= 0.923
	PREFLASHED	= 0.975

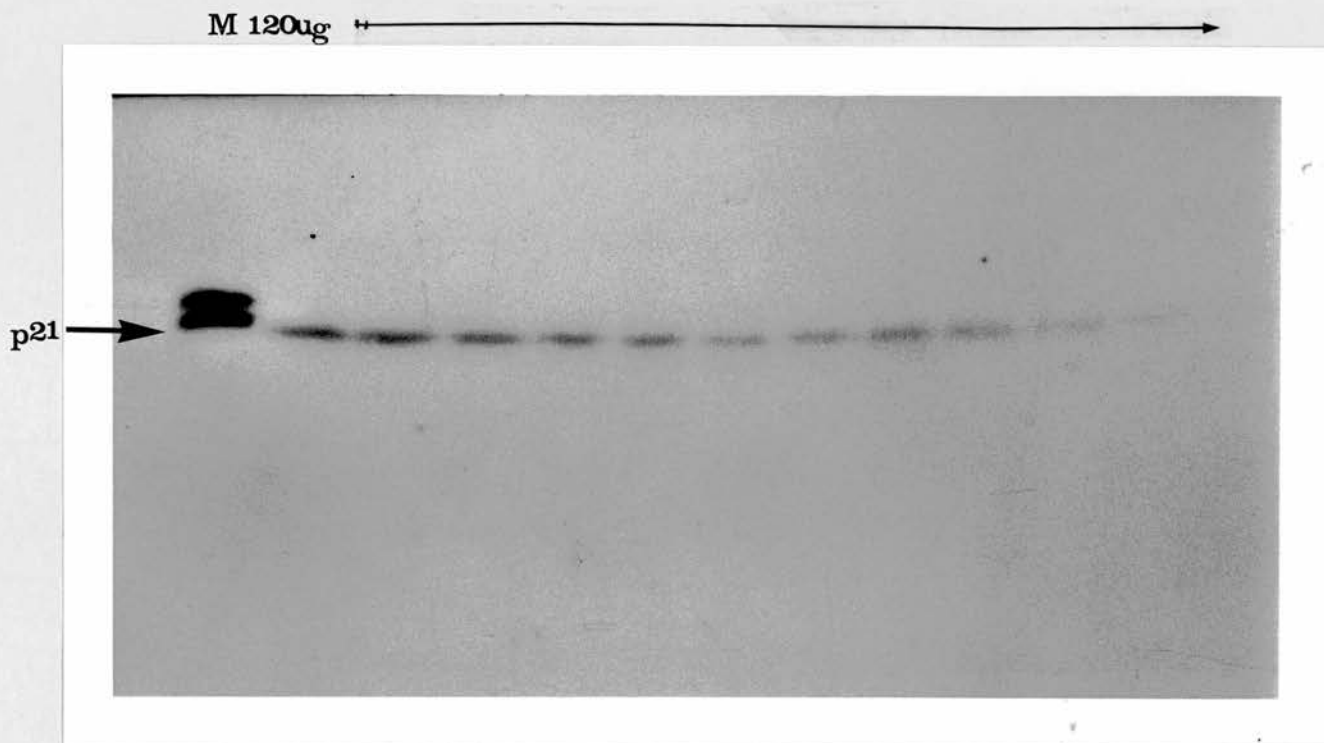


Plate 1:

A representative Western blot for p21 with the Y13-259 monoclonal with a serial diluted MCF-7 cell lysate.

An MCF-7 cell lysate (section 2.3.1) was loaded at decreasing concentrations of protein from 120 to 10 μ g (decreasing by 10 μ g per lane).

M- lysate prepared from NIH/3T3 *v-ras* transformed cells (20 μ g of protein loaded).

3.6 ALTERNATIVE METHODS OF QUANTIFYING WESTERNS

3.6.1 Introduction:

As demonstrated in section 3.4 the amount of soluble protein extracted from MCF-7 cells was related to the cell number. This suggests that loading of protein from different samples of an individual cell line, a direct comparison of the amount of p21 would be possible. However, this assumes that the extraction of soluble protein for each sample of cells is consistent. To ensure that protein content can be related to cell number for each experiment it would be necessary to construct a standard calibration plot of protein concentration versus cell number for each sample (eg. control cells, drug treated cells, etc.). Due to limited amounts of cellular material during experiments this is not always possible. Alternatively, an internal standard could be measured, preferably a protein that is extracted with the same efficiency as p21. Hypothetically, a number of possibilities do exist, such as cytoskeletal proteins and chromatin. However, if these proteins are to be considered as internal standards then they must be expressed at a constant level in the presence of different agents. Actin was considered a good candidate since mRNA analysis of *ras* oncogene has been studied normalised to constitutively expressed actin mRNA (Bignami *et al.*, 1988). Other alternatives considered were tubulin, cytokeratin and acid soluble histones.

3.6.2 Actin and Tubulin Proteins:

To detect the level of actin a polyclonal antibody was used, and for tubulin both a polyclonal and a monoclonal antibody were used in Western blotting (section 2.6.1). The appropriate IgG,

raised against the host species, for the primary antibody was also used. Rainbow™ Protein Molecular Weight Markers (200 - 14.3 kDa) contain proteins of known molecular weights coupled to dyes with specific colours. These proteins were used to indicate where the blot could be divided into two parts to give sections of different molecular weights; one section would contain the 21 kDa proteins and the other part the higher molecular weights of tubulin (55 kDa) and actin (42 kDa). Each section of the blot was then incubated with the appropriate antibodies (section 2.6.1).

To confirm that staining with these antibodies could be used as internal standards for quantifying p21, at least three criteria must be satisfied:

1. There is a linear relationship with respect to intensity of signal on X-ray film versus quantity of protein present (see section 3.5).
2. The ratio of p21 : INTERNAL STANDARD produce a constant value for varying concentrations of loaded protein on the blot.
3. The amount of protein is unchanged by drug treatment.

To address these issues soluble protein from a single MCF-7 cell lysate (section 2.3.1) was loaded in decreasing amounts of protein on a 12% SDS-PAGE gel and run (sections 2.5.1 and 2.5.2). The gel was blotted (section 2.5.5) and cut into two sections; one section contained proteins less than 30 kDa and the other section greater than 30 kDa. The higher molecular weight section of the blot was incubated with tubulin and actin polyclonal antibodies and the lower section with Y13-259 for p21 (section 2.6.1). The resulting blot was exposed to preflashed X-ray film (section 2.8.1) and scanned (section 2.8.2).

Plate 2 shows that the intensity of signal specific for p21 decreased with respect to the decreasing concentration of loaded protein on the blot (as previously demonstrated in section 3.5). This, however, was not the case for actin or tubulin staining. The tubulin signal appeared to increase as the protein concentration decreased, irrespective of whether or not the antibody was polyclonal or monoclonal. The actin staining was inconclusive because the signal was too weak. A linear relationship, as described for p21, for intensity of signal against the concentration of protein of an MCF-7 lysate could not be demonstrated for actin or tubulin.

However, the lysate produced from the NIH/3T3 *v-ras* transformed cell line used as a marker for p21 had a strong staining for tubulin using the monoclonal antibody. Plate 3 shows a Western blot of decreasing protein concentration of this lysate for p21 and tubulin. As demonstrated by this result, a relationship between tubulin staining with respect to protein concentration does exist. Using the scan data for the tubulin signal and the p21 signal calculations were made to determine if a constant ratio existed between the signals. A constant value for the ratio of p21 : tubulin signal intensity was evident for the first five protein concentrations but only if the \log_{10} of the scan data for p21 and tubulin were taken (Table 2):

$$\Rightarrow \log_{10}(\text{tubulin}) : \log_{10}(\text{p21})$$

These data demonstrate that tubulin in an NIH/3T3 *v-ras* cell lysate could be used to monitor the extraction of p21. However, this anti-tubulin antibody does not appear to work for the MCF-7

cell line. A hypothetical explanation for this difference may be antibody tissue type specificity. Cell type specificity of antibodies has been observed for the cytoskeletal protein myosin (Smith *et al.*, 1983). Antibodies raised against myosin from one cell type can cross-react with myosin from the same cell type of other species. However, cross-reactivity of the antibody to other cell types from the original animal is poor.

3.6.3 Cytokeratin 19:

The monoclonal antibody BA17 stains a cytokeratin species with a molecular weight of 19 kDa (staining of MCF-7 cytokeratin 19 has been previously demonstrated by Dr Joyce Taylor-Papadimitriou, Imperial Cancer Research Fund, London; personal communication). This protein was considered another alternative as an internal standard for quantifying p21.

MCF-7 cells were plated in 75 cm² flasks before being treated for 120 hours with either oestradiol (10 nM), tamoxifen (1 µM) or retinoic acid (1 µM) (section 2.1.4). Cells were collected and lysates prepared for each sample (section 2.1.5 and 2.3.1). The pellets that remained after the 100 000 g centrifugation, during preparation of lysates, were used for extraction of histone proteins (section 2.3.2). Each sample was loaded on a 12% SDS-PAGE gel at two concentrations, 80 µg and 100 µg of soluble protein (section 2.5.1 and 2.5.2). After transfer of the gel to nitrocellulose (section 2.5.5), the blot was sectioned, and the high molecular weight section incubated with actin and tubulin polyclonal antibodies and the other section incubated with antibody for p21 and cytokeratin 19 (section 2.6.1).

Plate 4 shows that staining with BA17 antibody of control lysates (incubated in the absence of drug) produced a very faint stain of at least two bands; one with an approximate molecular weight of 19 kDa and the other approximately 13 kDa. Interestingly, addition of tamoxifen to MCF-7 cells resulted in the appearance of three bands, two of which corresponded to those of the control but with a more intense stain. Retinoic acid treatment also resulted in staining of two bands corresponding to the control bands, as with tamoxifen, the signal was more intense. Oestradiol treatment of MCF-7 cells did not increase the signal intensity of these proteins and the staining pattern resembled the control.

It is not known if these bands are all related to cytokeratin 19 or if they are proteins with the same epitope detected by BA17. Nevertheless, these data suggest that tamoxifen and retinoic acid treatment of MCF-7 cells alters the expression of different BA17 detectable proteins compared to untreated cells. The observation that drug treatment can preferentially alter expression of BA17 detectable proteins makes this antibody unsuitable for staining an internal standard for p21 extraction.

3.6.4 HISTONES:

Histones are acid soluble proteins that bind to genomic DNA to form chromatin. These proteins are not soluble in Buffer 10 with the result that they are pelleted during the 100 000 g centrifugation. These proteins were extracted from the pellet as described in section 2.3.2.

Histone extracts prepared from the sample pellets from the above experiment (section 3.6.3) were loaded and run through a 12 % SDS-PAGE gel (section 2.5.1 & 2.5.2). Samples of commercially prepared histone extracts were also prepared as detailed for the histone extraction from pellets (section 2.3.2) and resolved by SDS-PAGE gel. The gel was stained according to the method for Coomassie blue staining (section 2.5.4). The stained gel, shown by Plate 5, shows that the majority of the proteins extracted from the pellet had similar mobilities on SDS-PAGE gel as the major protein species in the commercial preparations of histones. The Coomassie stained gels were scanned in the same way the X-rays were scanned (section 2.8.2).

No relationship was found between histone staining and p21. It is possible that extraction of histone from the pellets may not be consistent.

3.6.5 Protein:

Hypothetically, it would be more advantageous to measure a protein of interest in an extract by normalising the result to an internal standard. For example, RNA measurements (Northern Analysis) are usually not normalised to quantity of loaded RNA because of possible degradation of the sample but normalised to, for example, actin mRNA that should degrade at the same rate as the RNA of interest, therefore giving an internal standard for detectable RNA. The Bradford Assay (Bradford, 1976) is based on the observation that the absorbance of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when it binds to protein. The concentration of protein is determined from the absorbance of G-250 bound to protein at 595 nm. For example, determination of a

protein concentration (10 mg/ml) by the Bradford assay for solutions each containing a single protein of different molecular weights, including thyroglobulin, catalase or lysozyme would yield similar results of approximately 10 mg/ml (Bio-Rad Protein Assay handbook, p.4; BIO-RAD Laboratories). In other words, if a lysate with 1 mg/ml of soluble protein on analysis then partially degraded, it could still give the same result when measured again giving no indication of degradation. This suggests that if two identical samples had a fixed amount of protein loaded on a gel, the quantity of protein resolved may not be the same if one of those samples had partially degraded, possibly affecting the staining of a specific protein.

To guard against this gels could be Coomassie stained and the staining intensity of each sample compared. However, transfer of stained proteins to nitrocellulose is impaired (Stott, 1989). Naphthol blue black can stain protein bound to nitrocellulose. This can then be visually checked for differences in protein staining per sample. However, differences of 10 µg per sample are difficult to see.

Attempts to normalise the p21 signal with actin, tubulin, cytokeratin 19 and histone did not prove to be successful for this particular study. It was therefore decided that p21 data would be normalised to the amount of loaded protein. To reduce the level of protein degradation, lysates were prepared at 4 °C and freezing and thawing kept to a minimum. In addition, blots were stained for total protein using naphthol blue black (section 2.6.3) to ensure that each lane visually appeared to have similar amounts.

M

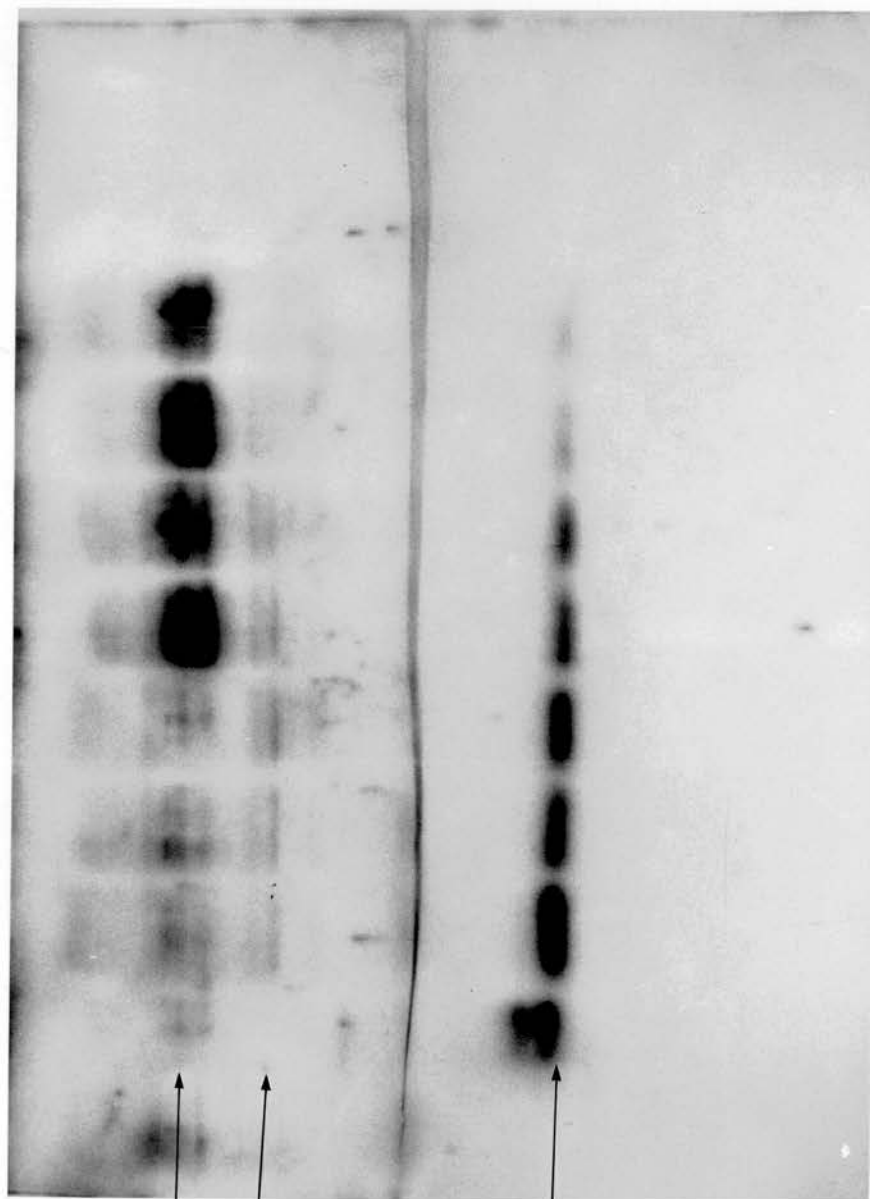


Plate 2:

A representative Western blot of an MCF-7 cell lysate loaded with decreasing concentration of protein and immunoblotted for tubulin (T), actin (A) and p21.

M- NIH/3T3 v-*ras* transformed cell lysate

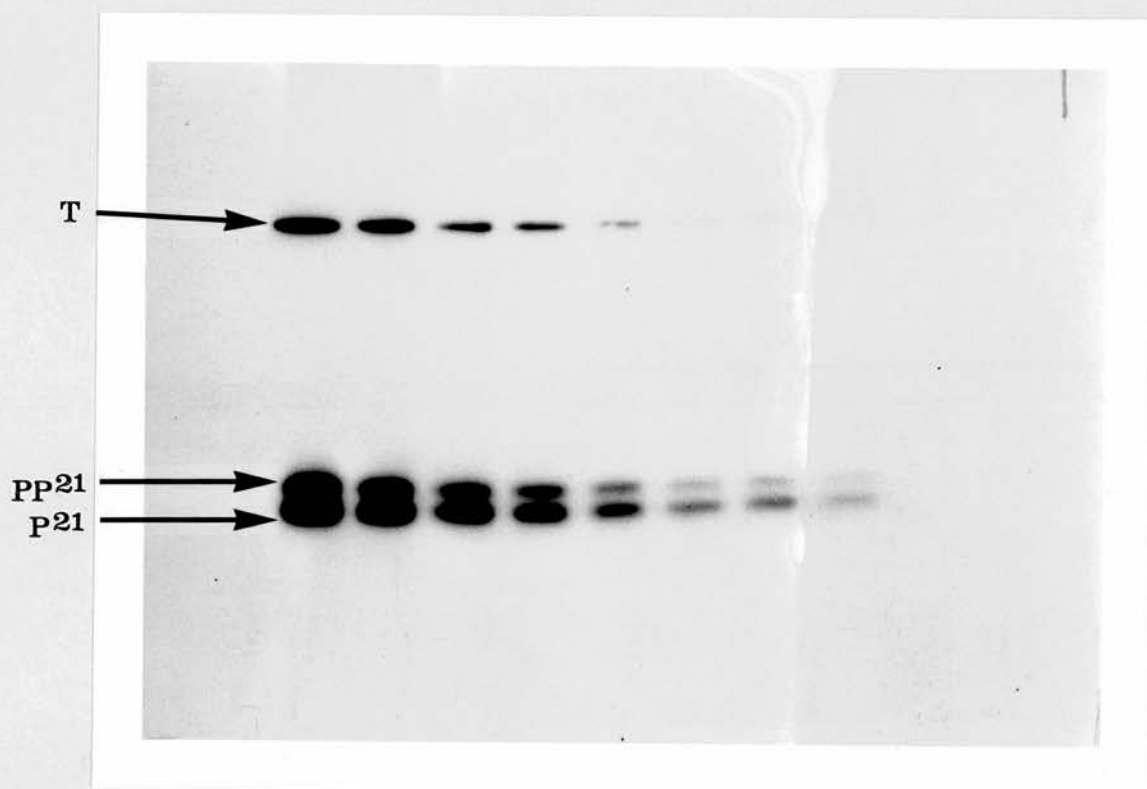


Plate 3:

A representative Western blot of an NIH/3T3 *v-ras* cell lysate loaded with decreasing concentrations of protein and immunoblotted for **p21**, phosphorylated p21 (**pp21**) and tubulin (**T**).

Protein ($\mu\text{g/ml}$)	50	37.5	28.1	21.1	15.8	11.9	8.9	6.7
1. Tubulin	88.1	70.1	34.0	28.4	16.9	4.1	3.2	3.0
2. p21 viral cellular	193.9 261.1	150.9 212.2	87.7 134.6	65.2 106.1	40.2 84.6	18.2 37.5	16.4 32.7	8.1 18.0
3. log Tubulin/ log p21 viral	0.80	0.80	0.79	0.80	0.77	0.50	0.40	0.50
4. log Tubulin/ log p21 cellular	0.85	0.80	0.72	0.72	0.64	0.40	0.33	0.38

TABLE 2:

Scan data produced from a Western blot of a lysate of NIH/3T3 *v-ras* transfected cells loaded at different protein concentrations and immunoblotted for p21 and tubulin.

Scan data derived from plate 3

1. Signal intensity for tubulin stained with the mouse monoclonal.
2. Signal intensity for phosphorylated viral p21 and cellular p21 stained with Y13-259 monoclonal.
3. Ratio of $\log_{10}(\text{tubulin})$: $\log_{10}(\text{viral p21})$
4. Ratio of $\log_{10}(\text{tubulin})$: $\log_{10}(\text{cellular p21})$

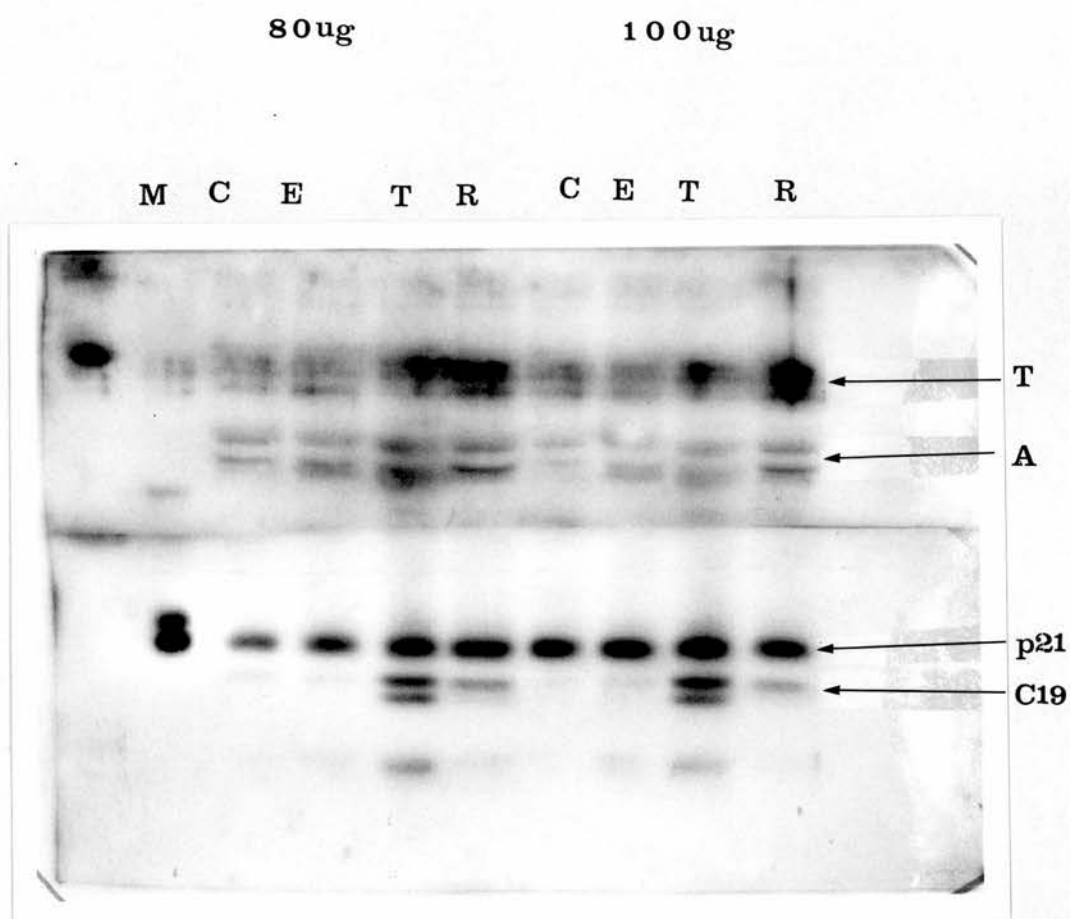


Plate 4:

A representative Western blot of MCF-7 cells, treated with drug for 120 hours before being prepared as a lysate, immunoblotted for tubulin (T), actin (A), p21 and cytokeratin 19 (C19).

MCF-7 cells were culture for 120 hours either in the absence (control; C) or the presence of oestradiol (10 nM; E), tamoxifen (1μM; T) or retinoic acid (1μM; R).

Two sets of concentrations were loaded for each sample; 80 μg and 100 μg.

M- NIH/3T3 v-ras cell lysate

M H_I H_{III} H_V 1 2 3 4

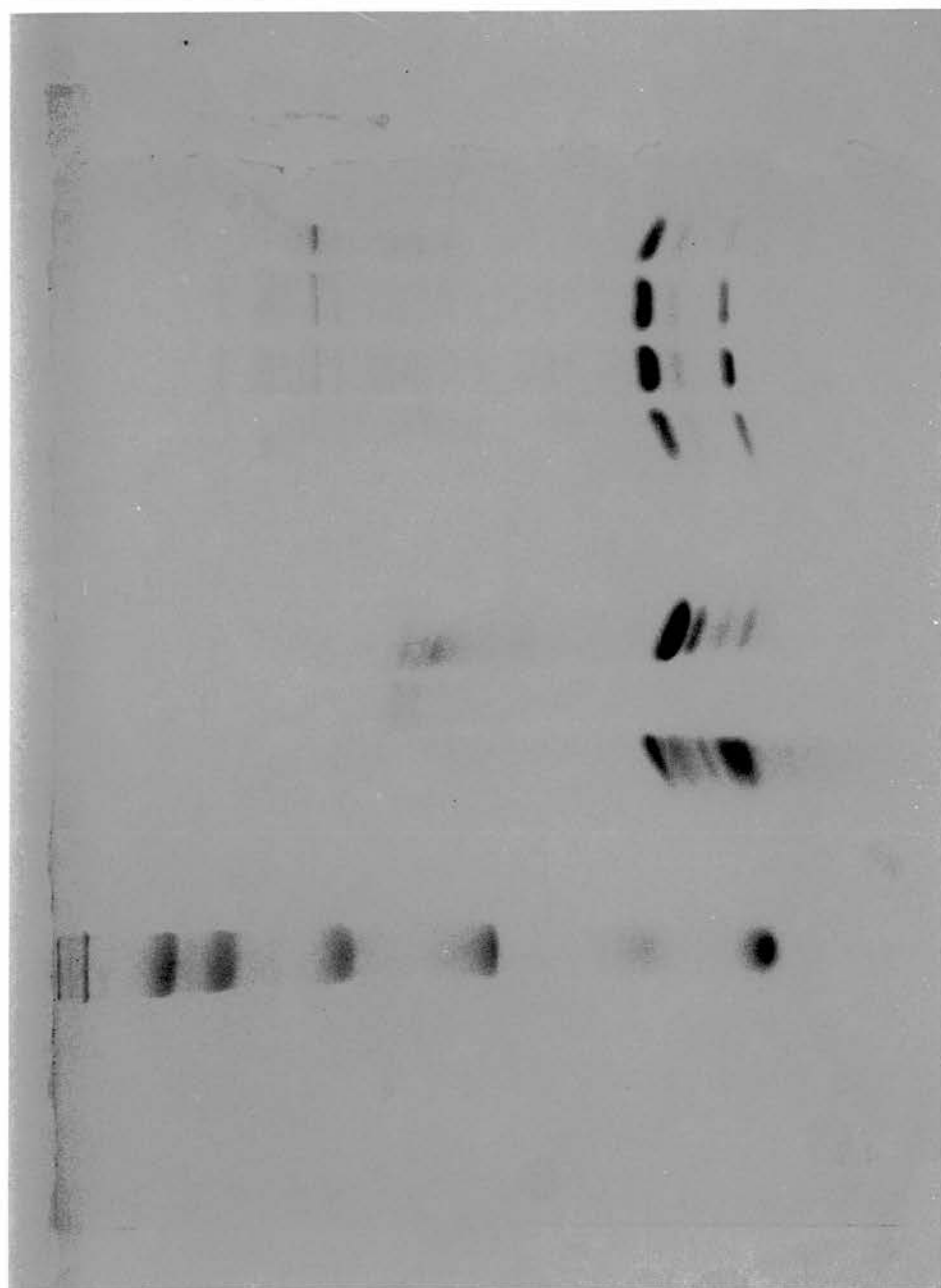


Plate 5:

A Coomassie blue stained 12% SDS-PAGE gel of commercial histone preparations and acid soluble extracts from MCF-7 cell lysate pellets. Samples 1 to 4 were prepared from the lysate pellets of H_I, H_{III} and H_V were loaded at 10 µg per lane. Samples 1 to 4 were prepared from the lysate pellets of MCF-7 extracts used in section 3.6.3 (photograph 4) and loaded at 50 µg per lane.

3.7 EFFECT OF 8-Cl cAMP ON THE EXPRESSION LEVEL OF p21

To establish if the growth inhibitory response by 8-Cl cAMP in DMEM containing 10% FCS (v/v) was associated with a change in p21 expression levels, MCF-7 cells were plated in 175 cm² flasks and treated with oestradiol (10 nM), 8-Cl cAMP (10 μ M) or oestradiol and 8-Cl cAMP for 120 hours (section 2.1.4). After this period cells were collected from the flasks (section 2.1.5) and prepared into lysates (section 2.3.1). A fixed quantity of soluble protein (100 μ g and 50 μ g), as determined by the Bradford assay (section 2.4), was loaded for each sample on to a 12% SDS-PAGE gel and resolved (sections 2.5.1 & 2.5.2). Western blotting was performed for p21 according to section 2.6.1. The blot was then exposed to X-ray film (section 2.8.1) and the signal quantified by scanning (section 2.8.2). After developing, the blots were finally stained with naphthol blue black (section 2.6.3) to check that the general staining intensity for protein in each lane appeared similar.

Plate 6 shows a representative blot for this experiment. The staining for p21 was very specific with virtually no visible background staining. The intensity of the signals also appeared very similar with the naked eye. Table 3 contains the accumulative scanning data from all the experiments; the scan data is presented as a percentage of the control data (i.e. 100%). The p21 expression in MCF-7 cells after culture in DMEM containing 10% FCS (v/v) did not appear to be affected by 8-Cl cAMP even though the cells were growth inhibited after the five day exposure. Treatment with oestradiol produced a very small

increase in expression level of p21 by 1.3 fold over the control. However, this increase was possibly not significant due to a high value of 38.6% for the standard deviation. Experiments in which MCF-7 cells were cultured with DMEM containing 1% FCS (v/v) produced similar results as for 10% FCS conditions (data not shown).

Tagliaferri *et al.* (1988) and Katsaros *et al.* (1988) studied the effects of 8-Cl cAMP on expression of p21 after three days. This time period was also investigated with MCF-7 cells in DMEM containing 10% FCS (v/v) with 8-Cl cAMP (10 μ M), and again produced a similar result as for the five day exposure (data not shown).

These MCF-7 cells treated with 8-Cl cAMP (10 μ M) did not appear to have reduced p21 expression, irrespective of the effect on cellular proliferation.

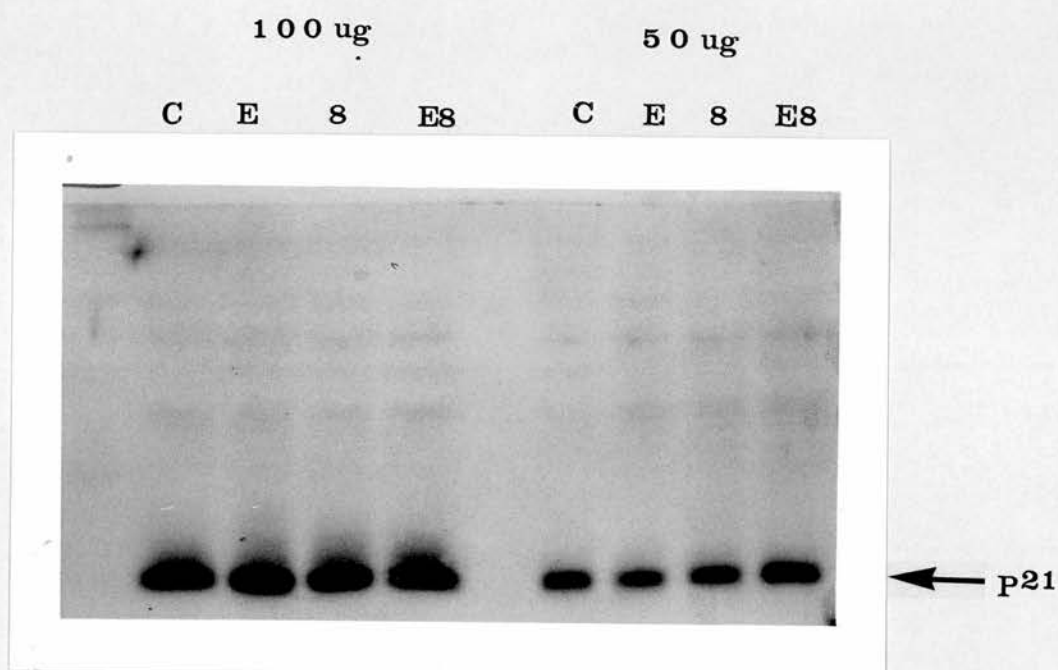


Plate 6:

A representative Western Blot of lysates, prepared from MCF-7 cells treated with drug for 120 hours and immunoblotted for p21.

C- control, no drug; **E-** oestradiol (10 nM); **8-** 8-Cl cAMP (10 μ M);

E8- oestradiol and 8-Cl cAMP

Each sample was loaded at two concentrations; 100 μ g and 50 μ g

<u>DRUG</u>	<u>% of CONTROL SIGNAL</u>
Oestradiol (10 nM) =	130% \pm 38.6
Oestradiol (10 nm) = + 8-Cl cAMP (10 μ M)	101% \pm 31.0
8-Cl cAMP (10 μ M) =	100% \pm 30.0

Table 3:

The expression level of p21 in MCF-7 cells exposed to drug for 120 hours.

The above data represents the average of seven experiments presented as a percentage of the control signal (i.e. 100%).

Errors are presented as standard deviations.

3.8 THE EFFECT OF 8-Cl cAMP ON THE EXPRESSION LEVELS OF THE cAMP-DEPENDENT PROTEIN KINASE REGULATORY SUBUNITS

Katsaros *et al.* (1988) correlated a change in the relative expression levels of the cAMP-dependent kinase regulatory subunits RI and RII with the growth inhibition of MCF-7 cells treated with 8-Cl cAMP, in the presence or absence of oestradiol. To determine whether 8-Cl cAMP (10 μ M) could mediate a change in RI and RII expression MCF-7 cells were treated with drug in DMEM containing 10% FCS (v/v) containing medium for 72 hours. Cells were collected after incubation and prepared in to cytosols (section 2.3.3). Two volumes of each cytosol were treated with [32 P] 8-azidoadenosine-3':5' cyclic monophosphate according to section 2.7 before being loaded (section 2.5.1) onto a 12% SDS-PAGE gel (section 2.5.2). The gel was dried (section 2.5.3) and exposed to X-ray film (section 2.8.1). The X-ray film was developed and the intensity of signal on the film measured by scanning (section 2.8.2).

The photoaffinity labelling of RI (49 kDa) and RII (55 kDa), shown in plate 7, was very specific with very little visual background. Each sample was loaded at two volumes; 75 μ l and 25 μ l. Table 4 shows the scan data expressed as a ratio for RI : RII. The oestradiol treated cells appeared to have a lower value for the RI : RII ratio which would indicate an increase in expression of RII, or a decrease in RI. Moreover, no apparent change in the expression ratio of the regulatory subunits was evident for cells treated with 8-Cl cAMP.

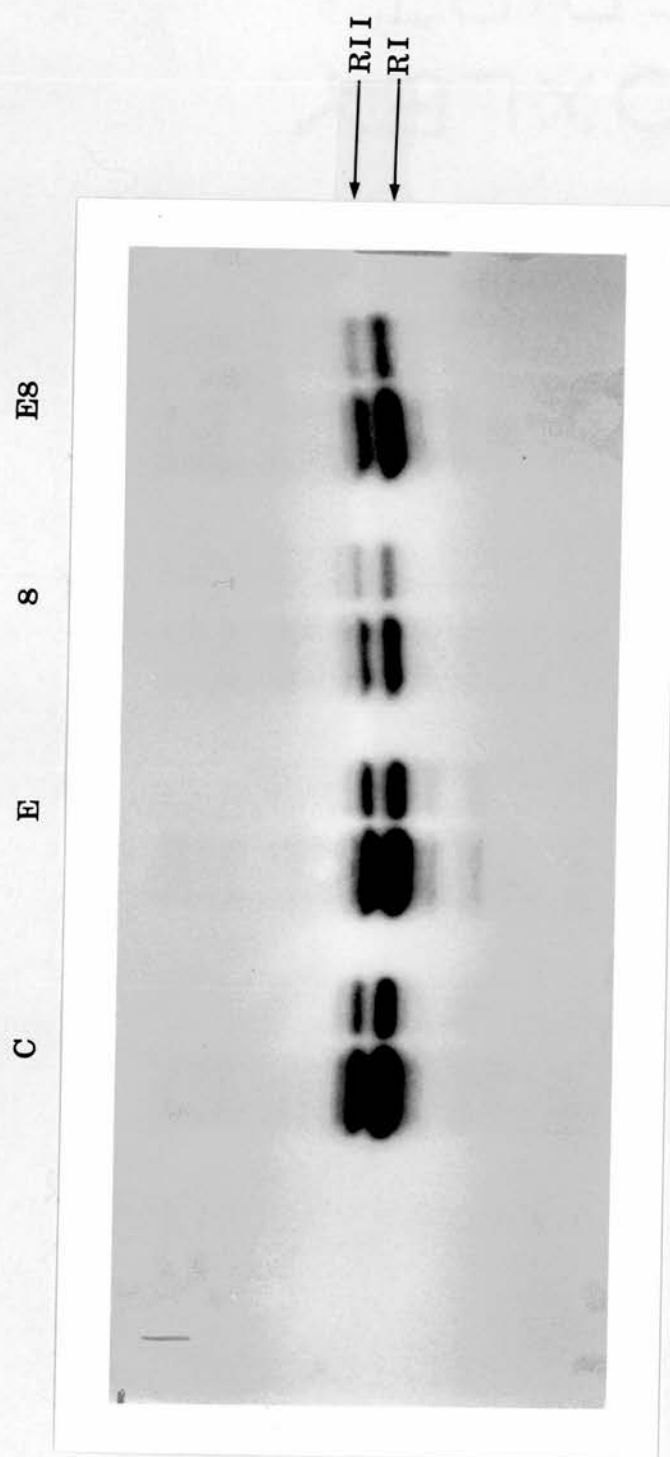


Plate 7:

Photoaffinity labelling of the cAMP-dependent protein kinase regulatory subunits (RI; 49 kDa, RII; 55 kDa) with [32 P] 8-azido cyclic AMP of cytosol prepared from MCF-7 cells treated with drug for 72 hours.

C- control, no drug; E- oestradiol (10 nM); 8- 8-Cl cAMP (10 μ M);

E8- oestradiol and 8-Cl cAMP

Each sample was loaded with 75 μ l and 25 μ l.

<u>DRUG</u>	<u>Ratio of RI : RII</u> (75µl / 25µl of cytosol)
Control	= 2.38 / 2.77
Oestradiol (10 nm)	= 1.69 / 1.71
8-Cl cAMP (10 µM)	= 2.93 / 2.62
Oestradiol + 8-Cl cAMP	= 1.74 / 1.90

Table 4:

The expression ratio of the RI and RII cAMP-dependent protein kinase regulatory subunits in MCF-7 cells treat with drug for 72 hours.

The above data represents the value for the ratio of RI : RII from the scanning data derived from plate 7. The two sets of data are for the same sample but different volumes were used for the assay.

3.9 CELL CYCLE ANALYSIS OF MCF-7 CELLS TREATED WITH 8-Cl cAMP

3.9.1 Introduction:

Tagliaferri *et al.* (1988) studied the effect of 8-Cl cAMP (10 μ M) on the cell cycle progression of MCF-7 cells cultured in 10% FCS conditions for three days. The authors showed that the inhibition of cell proliferation in the presence of 8-Cl cAMP did not correspond to any difference in cell cycle progression compared to untreated cells. This experiment was repeated using our MCF-7 cells to confirm if the growth inhibition observed with these cells by 8-Cl cAMP had an effect on cell cycle progression.

3.9.2 Cell Cycle Progression:

MCF-7 cells were cultured in 175 cm² flasks until the cells appeared to occupy 50% of the flask surface. The medium was replaced with DMEM containing 10% FCS (v/v) or HITS (section 2.2.1); a serum substitute (see section 3.11). 24 hours later, medium was replaced with fresh medium (control) or fresh medium with 8-Cl cAMP (10 μ M). After 72 hours the cells were harvested (section 2.5.1). The cell suspension was centrifuged at 750 g for 10 minutes at 4 °C to collect the cell pellets and then treated for flow cytometry analysis (section 2.10). Cells were analyzed using a FACScan flow cytometer (**Becton Dickinson**) and determination of the intensity of fluorescence of DNA bound propidium iodide dye versus cell number was calculated and peak areas determined. Three samples of 10⁶ cells per ml from each flask were analyzed and the average for each stage of the cell cycle determined.

The FACscan data in Table 5, shows that 8-Cl cAMP causes an accumulation of MCF-7 cells in the G2 + M cultured in DMEM containing 10% FCS (v/v). However, drug in HITS medium appears to have no effect on the cell cycle. The data is expressed as percentage of the control and plotted in Figure 6.

These data suggest that the growth inhibition of MCF-7 cells cultured in medium containing 10% FCS (v/v) may be associated with a blockage in G2 + M phase; this contradicts previous reports. No blockage by 8-Cl cAMP with MCF-7 cells cultured in HITS medium is consistent with the findings from section 3.11.3, that 8-Cl cAMP has no effect on proliferation in these conditions.

<u>Phase</u>	G0 + G1	S Phase	G2 + M
HITS	74.2 ± 0.9	18.4 ± 0.7	7.4 ± 0.8
HITS-8-Cl	75.8 ± 1.6	17.3 ± 3.5	7.0 ± 1.8
<u>% of control</u>	<u>102%</u>	<u>94%</u>	<u>95%</u>
10%	70.4 ± 1.5	26.0 ± 2.0	3.6 ± 0.7
10%-8-Cl	67.4 ± 1.4	19.9 ± 2.0	12.8 ± 0.8
<u>% of control</u>	<u>96%</u>	<u>77%</u>	<u>356%</u>

Table 5:

The effect of 8-Cl cAMP on the Cell cycle progression of MCF-7 cells incubated in DMEM containing either 10% FCS (v/v) or HITS for 72 hours in the absence (control) or presence of drug (10 µM). Data are the mean of triplicates ± standard deviation.

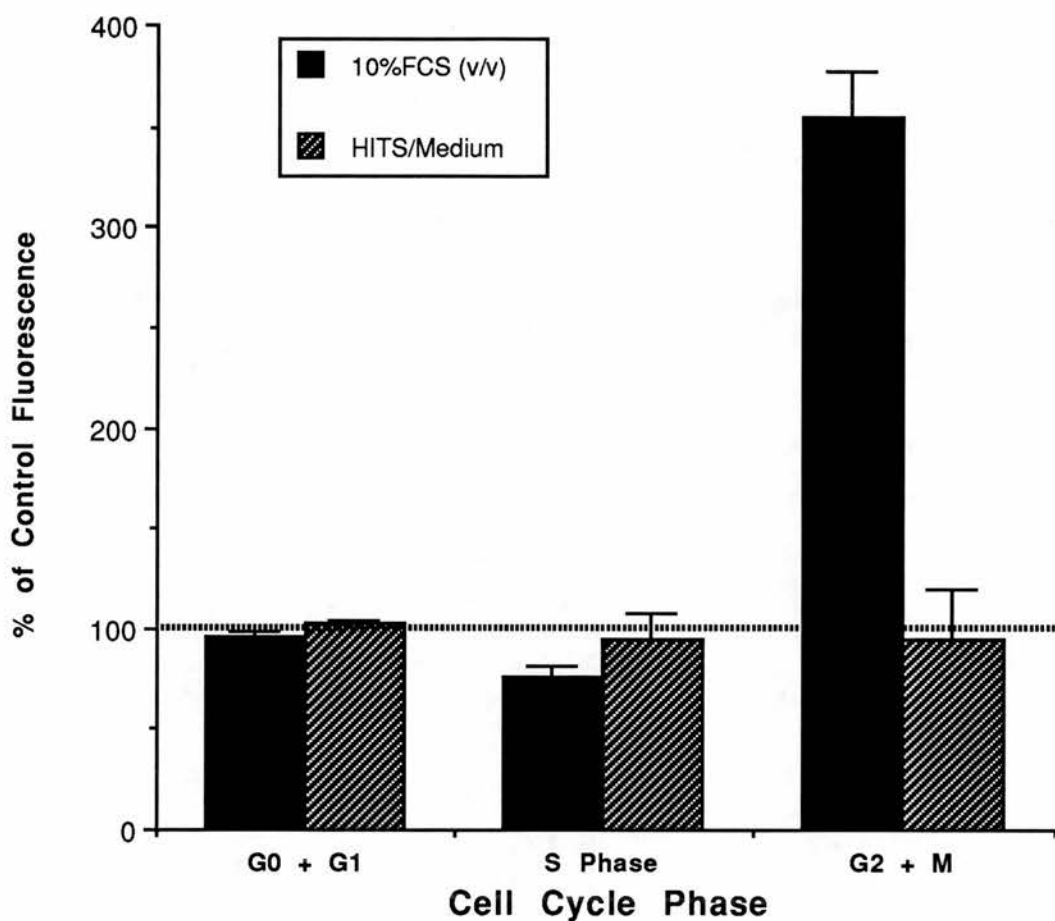


FIGURE 6:

The effect of 8-Cl cAMP (10 μ M) on the cell cycle progression of MCF-7 cells cultured for 72 hours in DMEM containing 10% FCS (v/v) or HITS.

Each bar is expressed as a percentage of the cell cycle phase relative to the control cells (no drug; 100%); values derived from Table 5.

Data are means of triplicate values \pm standard deviation.

3.10 SERUM-DEPENDENT 8-Cl cAMP INHIBITION OF MCF-7 CELLS

The only observation so far made with MCF-7 cells in this study with regards to 8-Cl cAMP (10 μ M) is growth inhibition. However, this response appears to be serum-dependent (section 3.3). To clarify the response, media containing a range of FCS concentrations were added to MCF-7 cells plated in 6 well plates (section 2.1.4) in the presence or absence (control) of 8-Cl cAMP (10 μ M). All plates were counted after a period of 120 hours (section 2.1.5).

Data presented in Figure 7 show that the control cell numbers increased slowly from 1% to 6% FCS (v/v). The arrow on the Figure indicates the number of cells present per well before addition of media. This would suggest that the lower concentrations of serum in the media do not produce similar basal growth levels. However, growth inhibition by 8-Cl cAMP appears to be less with 1% than for any other concentration of serum. Furthermore, the level of growth inhibition increased with 2% serum and a further, but smaller level in inhibition occurred with 3% - 5%. By 5% the level of growth inhibition plateaus, giving no further increase with increasing concentration of serum.

These data suggest that the serum-dependent growth inhibition of MCF-7 cells by 8-Cl cAMP may require a factor originating from serum.

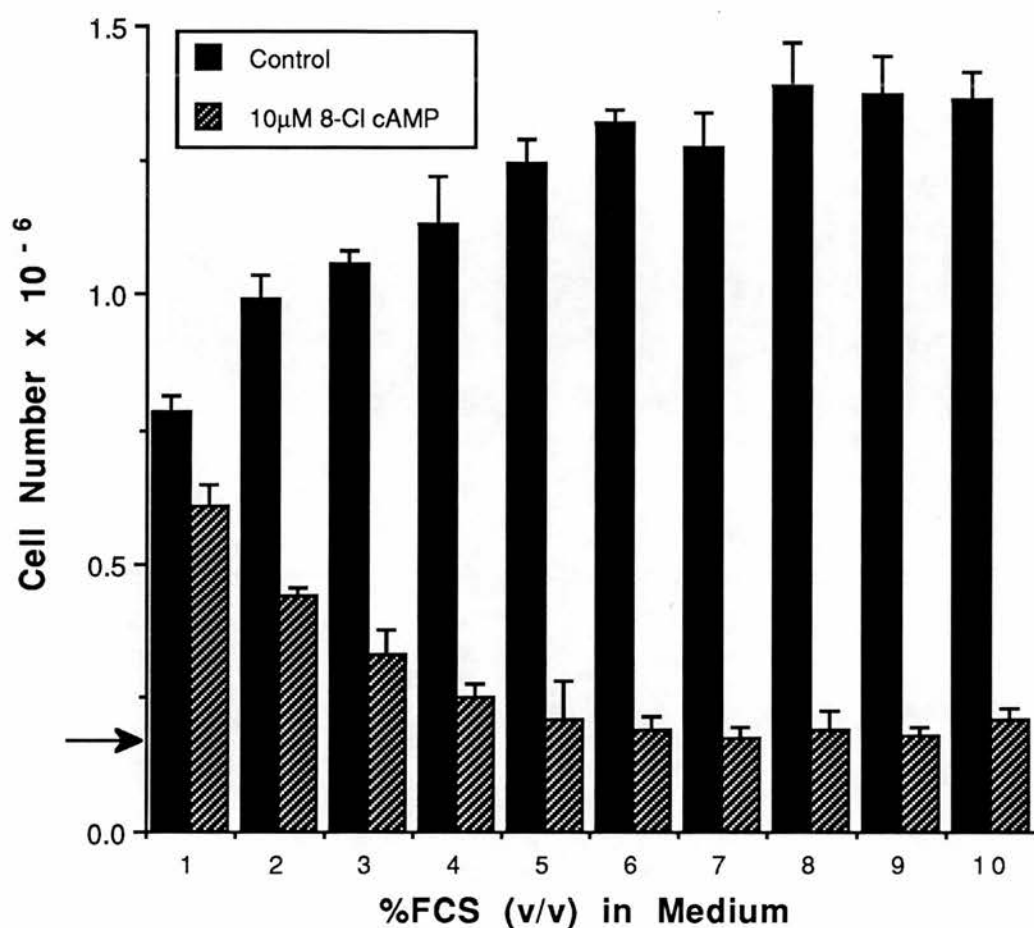


FIGURE 7:

The effect of 8-Cl cAMP (10 μ M) on growth of MCF-7 cells cultured for 120 hours in DMEM containing different concentrations of FCS (v/v).

Arrow indicates cell number on addition of drug.

Data are the means of triplicate values \pm standard deviation of a representative experiment.

3.11 USE OF SERUM-FREE MEDIUM TO STUDY THE INVOLVEMENT OF A PUTATIVE SERUM FACTOR DEPENDENT 8-Cl cAMP INHIBITION

3.11.1 Introduction:

To investigate this putative serum borne factor(s) as suggested by data from section 3.3 and 3.10 it would be advantageous to develop culture conditions that do not require the addition of serum to medium in order to maintain cellular proliferation. If the thesis is correct, in serum-free conditions MCF-7 cells would not respond to 8-Cl cAMP unless the factor(s) was added to the medium. Such a bio-system will enable the identification of potential factors.

MCF-7 cells have been successfully cultured in serum-free conditions containing EGF, insulin, transferrin and prostaglandins (Barnes & Sato, 1979). However, due to the expense of EGF and prostaglandin ($\text{PGF}_{2\alpha}$) this precluded the use of this serum-free medium. Hydrocortisone, insulin, transferrin, oestradiol and selenium (HITES) have been used in medium as a serum substitute for the growth of small cell lung carcinoma cell lines (Carney *et al.*, 1981). Furthermore, HITS has also been used successfully as a serum substitute for the growth of ovarian cancer cell lines including PEO1 and PEO14 (Personal communication with Simon Langdon, Imperial Cancer Research Fund, Edinburgh).

3.11.2 Serum-Dependent Inhibition Of PEO1 Cells By 8-Cl cAMP:

Inhibitory effects of 8-Cl cAMP on the ovarian cancer line PEO1 have not been reported. Since these cells can grow in serum-

free conditions a repeat of the previously described experiment (section 3.10) was set up using RPMI 1640 medium containing HITS and different concentrations of FCS. Cells were treated with or without (control) 8-Cl cAMP (10 μ M) for 120 hours before determining cell numbers.

Figure 8 shows that the control PEO1 cell number increased from a plating density of 10⁵ cells/well to approximately 3 x 10⁵ cells/well cultured in HITS medium only. This indicates that a basal growth level for these cells was present for HITS medium. Furthermore, these cells did not appear to respond to 8-Cl cAMP. However, 8-Cl cAMP was inhibitory to proliferation of PEO1 cells in the presence of FCS. A similar response as described above for MCF-7 cells applied; the level of inhibition increased with serum content, plateauing off by 3% serum.

These data demonstrate that PEO1 cells have a similar serum-dependent growth inhibition by 8-Cl cAMP as described for the MCF-7 cells.

3.11.3 Effect Of 8-Cl cAMP On MCF-7 Cells Cultured In Medium Containing HITS:

To determine if HITS could be used as a serum substitute for MCF-7 cells, and if addition of 8-Cl cAMP (10 μ M) had an effect on proliferation in these conditions, a full growth experiment was set up to study the effect of DMEM containing HITS in the presence or absence (control) of 8-Cl cAMP plus or minus oestradiol (see section 3.1 for description of set up).

Control MCF-7 cells (no drug) appeared to proliferate with a doubling time of 2 days (Figure 9(a)). This doubling time was equivalent to the time for MCF-7 cells cultured in DMEM containing 1% charcoal-stripped FCS (v/v) in the presence of oestradiol (10^{-8} M) (section 3.1). These cells cultured in HITS medium do not appear to be stimulated by oestradiol. Furthermore, 8-Cl cAMP (10 μ M) does not appear to affect growth.

These data demonstrate that the serum-free HITS medium is suitable for maintaining proliferation of MCF-7 cells during culture for approximately 7 days. This proliferation is also unaffected by 8-Cl cAMP due to the absence of serum.

3.11.4 Effect of 8-Cl cAMP On The Growth Of MCF-7 Cells Cultured In HITS Medium Containing FCS:

To confirm that the serum-dependent 8-Cl cAMP inhibition of MCF-7 cells can still be demonstrated in DMEM containing HITS (HITS medium) a repeat of the PEO1 experiment described in section 3.11.2 was repeated.

Again, 8-Cl cAMP appeared to unaffected the proliferation of MCF-7 cells cultured in HITS medium in the absence of serum (Figure 9(b)). This result appears similar to the data presented in section 3.10 (Figure 7); the basal growth rate (control) increased with the concentration of serum. Furthermore, the level of 8-Cl cAMP growth inhibition increased with the concentration of serum, plateauing off by 3% serum.

These data demonstrate that the presence of HITS in the medium did not affect the serum-dependent 8-Cl cAMP growth

inhibition of MCF-7 cells. Furthermore, HITS medium in the presence of 8-Cl cAMP (10 μ M) had no inhibitory affect on cellular proliferation.

3.11.5 Advantage Of HITS In Medium:

To study the effect of an antiproliferative drug like 8-Cl cAMP, the drug needs to be added to viable, proliferating cells. If control cells do not proliferate in the culture conditions then addition of an antiproliferating agent would appear to have no effect. Addition of HITS to culture medium, therefore, maintains a level of proliferation in PEO1 cells and MCF-7 cells which enables one to make the conclusion from the above data that 8-Cl cAMP has no growth inhibitory affect on cells cultured in the absence of serum. Because a basal growth level can be maintained in HITS medium in the absence of serum, data from the above experiment (section 3.11.3) can now be expressed as a percentage of control growth (Figure 9(c)); each bar is represented as a percentage of cell numbers drug treated with respect to control cell numbers (no drug; 100%).

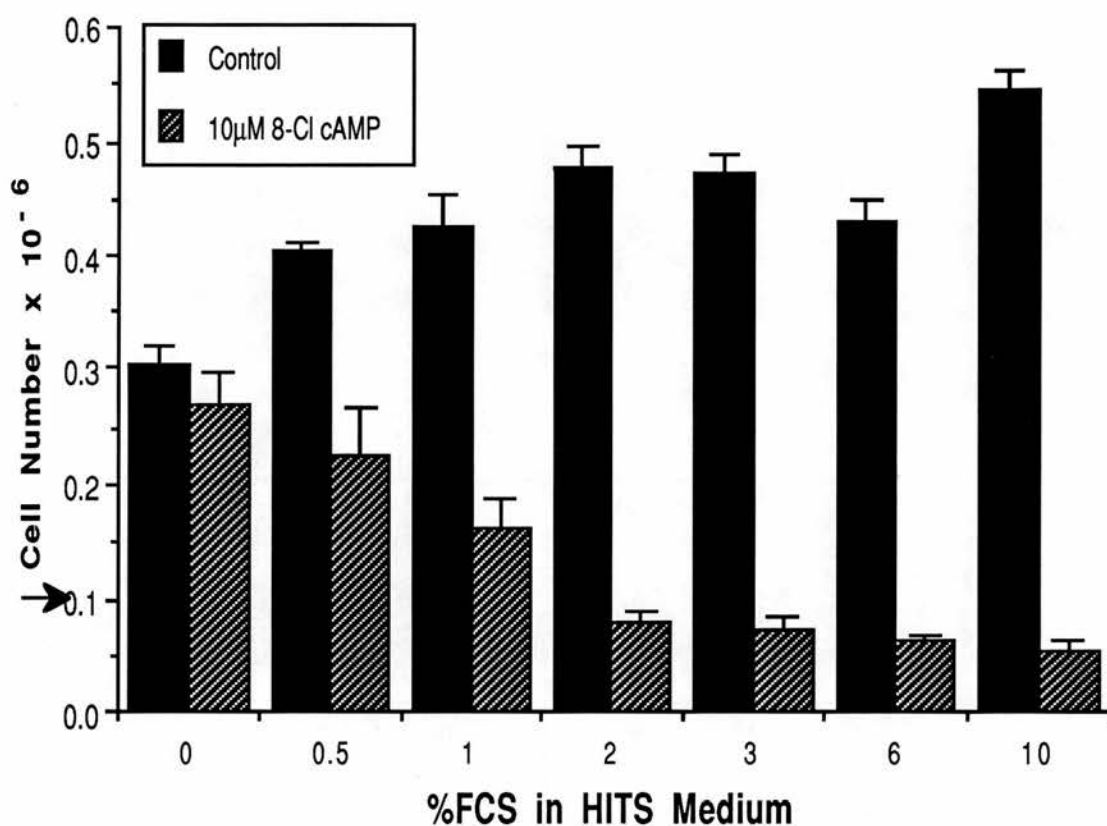


FIGURE 8:

The effect of 8-Cl cAMP (10 μM) on the growth of PEO1 cells cultured for 120 hours in RPMI containing HITS and different concentrations of FCS (v/v).

Arrow indicates cell number on addition of drug.

Data are the means of triplicate values \pm standard deviation of a representative experiment.

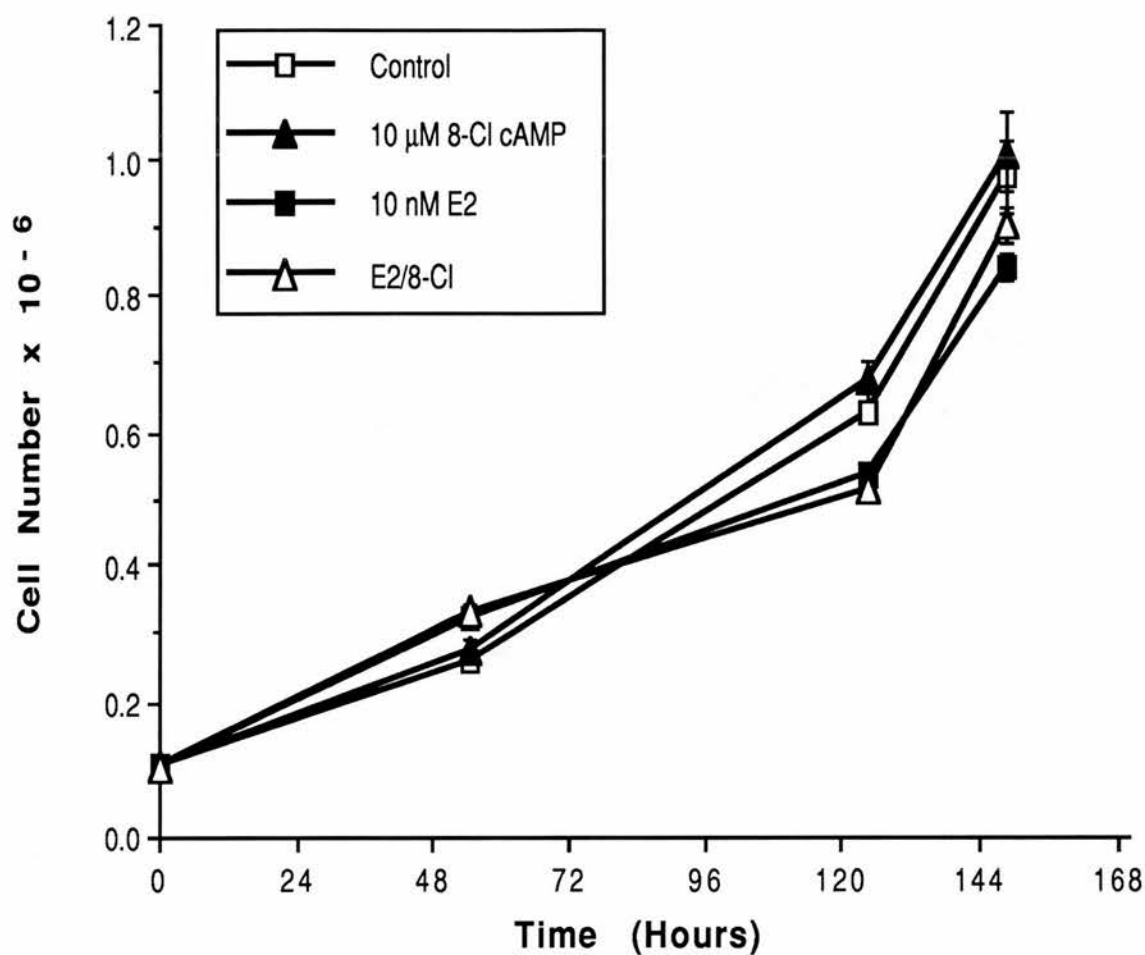


Figure 9(a):

The effect of oestradiol (**E2**; 10 nM), 8-Cl cAMP (**8-Cl**; 10 μM) and combination of oestradiol and 8-Cl cAMP (**E2/8-Cl**) on growth of MCF-7 cells cultured in DMEM containing HITS (sections 2.14, 2.1.5 and 2.2.1).

Data are the means of triplicate values \pm standard deviation of a representative experiment

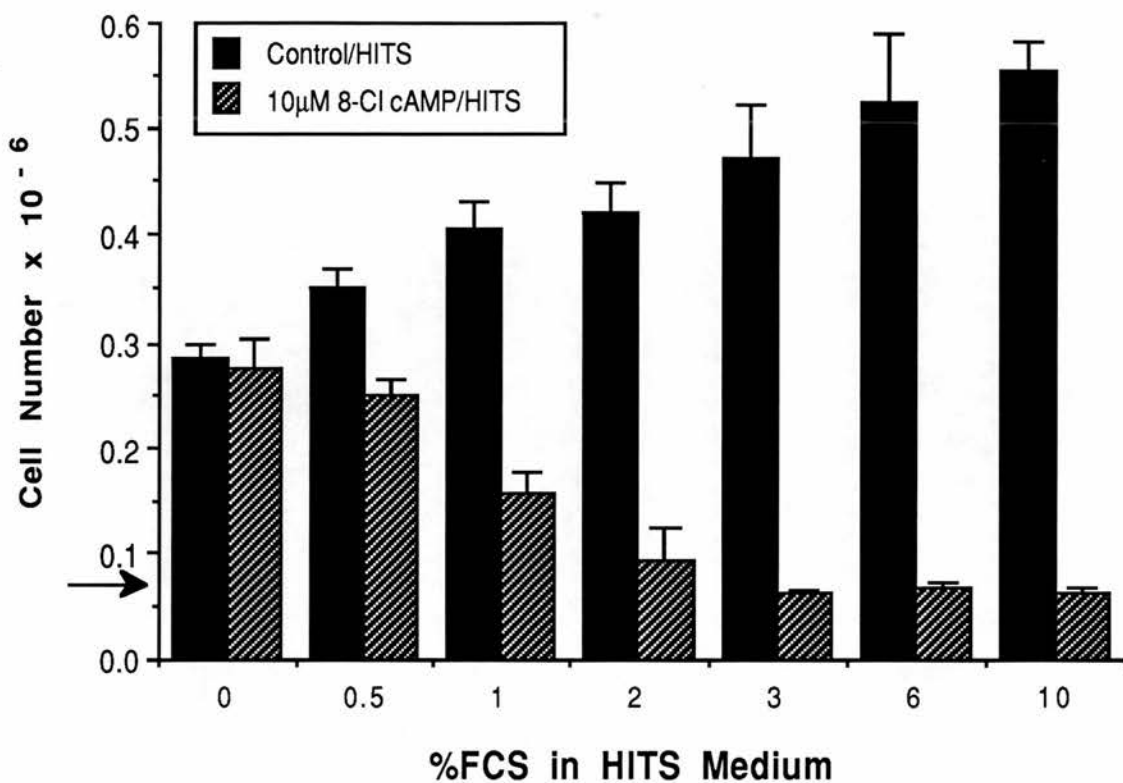


Figure 9(b):

The effect of 8-Cl cAMP (10 μ M) on the growth of MCF-7 cells treated for 120 hours in DMEM containing HITS and different concentrations of FCS (v/v).

Arrow indicates cell number on addition of drug.

Data are the means of triplicate values \pm standard deviations of a representative experiment.

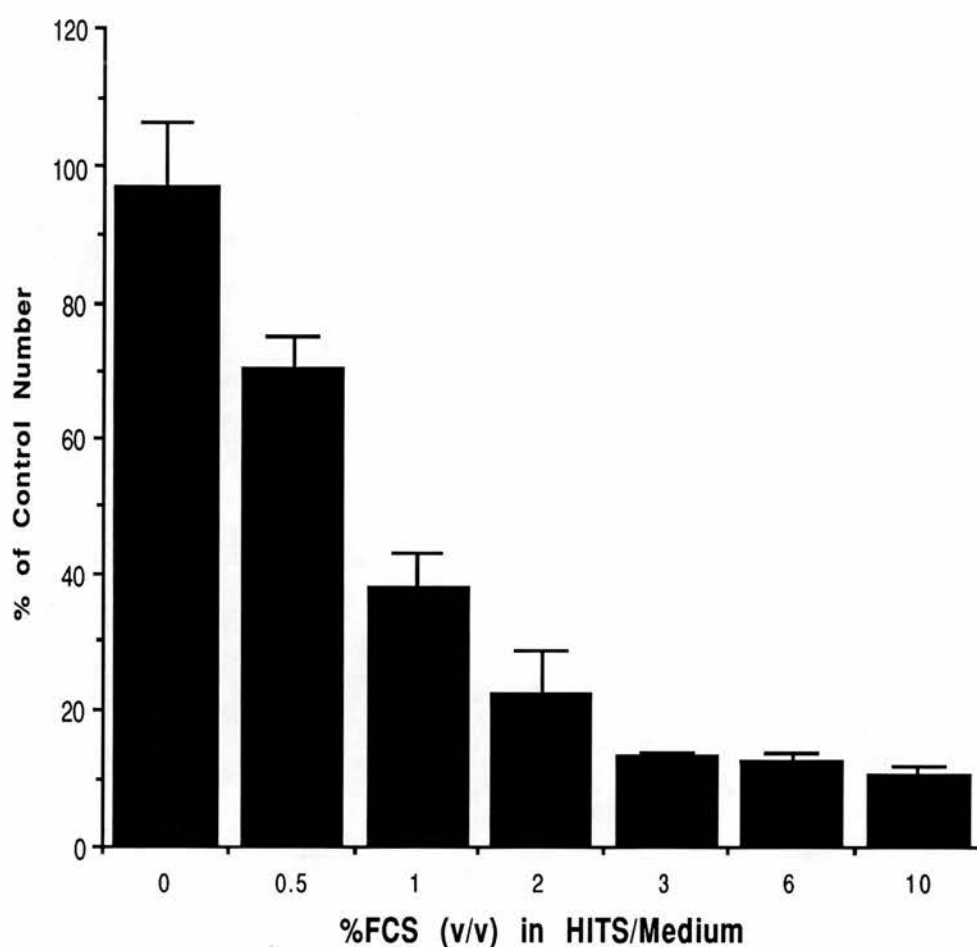


FIGURE 9(c):

The effect of 8-Cl cAMP (10μM) on the growth of MCF-7 cells treated for 120 hours in DMEM containing HITS and different concentrations of FCS (v/v).

Data are expressed as a percentage of control cells (not treated with drug) of triplicates \pm standard deviation.

3.12 OTHER CELL LINES HAVE A SERUM DEPENDENCE FOR 8-Cl cAMP INHIBITION

To determine if the serum-dependent 8-Cl cAMP growth inhibition demonstrated in MCF-7 and PEO1 cells extended to other cell lines the following were screened: human ovarian carcinoma cell line PEO4, hormone-unresponsive human breast cancer MDA-MB-231 and the colorectal carcinoma line HT-29. Cell lines were set up according to the experiment described in sections 3.11.4 and 3.11.2; cell lines were plated at 10^5 cells per well, except HT-29 which was plated at 10^4 cell/well. The cells were incubated with the appropriate HITS medium (MCF-7, MDA and HT-29 cultured with DMEM; PEO1 and PEO4 cultured with RPMI 1640) plus or minus (control) 8-Cl cAMP ($10\mu\text{M}$) and HITS medium containing 10% FCS (v/v) plus or minus (control) 8-Cl cAMP ($10\mu\text{M}$) for 120 hours before counting cells (section 2.1.5).

Data in Figure 10 is presented as a percentage of cell number for wells with 8-Cl cAMP against the control numbers (no drug). All cell lines cultured in HITS medium for 120 hours had an increase in cell number above the number of cells per well at time of drug addition. This indicated that for these cell lines, HITS medium maintained a level of proliferation for the period of incubation. No appreciable change in cell number was observed on addition of 8-Cl cAMP with any of the cell lines cultured in HITS medium. However, in the presence of 10% serum there was growth inhibition. The level of growth inhibition for $10\mu\text{M}$ 8-Cl cAMP varied for the different cell lines. PEO4, PEO1 and MCF-7 were inhibited the most followed by MDA-MB-231 cells and then HT-29 cells.

These data suggest that for these cell lines the presence of serum is also required for 8-Cl cAMP to affect growth.

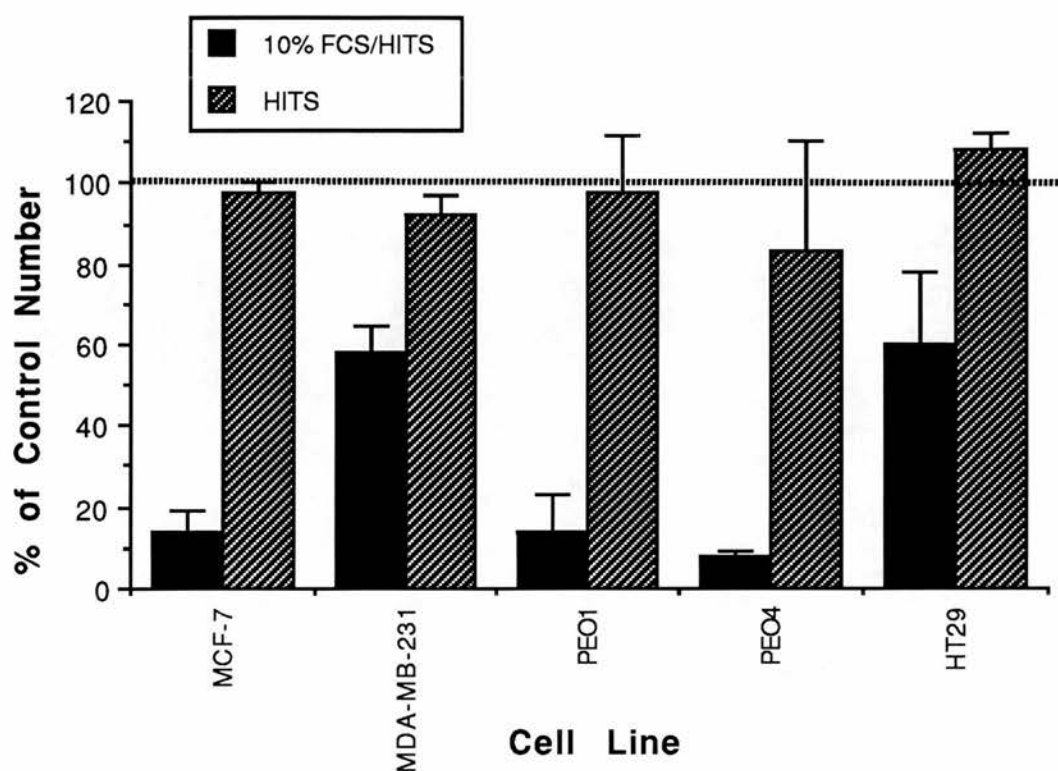


FIGURE 10:

The effect of 8-Cl cAMP (10 μ M) on the growth of different cell lines cultured for 120 hours in medium containing 10% FCS (v/v) (**10% FCS/HITS**) or **HITS**.

Cell numbers are expressed as a percentage of control cells (i.e. no drug; 100%) of triplicates \pm standard deviation.

3.13 THYMIDINE UPTAKE ASSAY

The experiments studied in the previous section utilize an assay involving a five day incubation in 6 well plates. Cell number was determined by harvesting each well and counting the cells with a Coulter counter. This is a very time consuming process and therefore limits the number of samples that can be processed. To enable faster assay and to increase the number of samples handled a 96 well plate assay was developed using MCF-7 cells. A repeat of the experiment described in section 3.11.3 was set up but using the level of [methyl-³H] thymidine incorporated into cellular DNA as a measure of cellular proliferation (section 2.1.6).

The CPM for 8-Cl cAMP (10 μ M) treated cells is expressed as percentage of the control (no drug) in Figure 11. Comparison of Figure 9(c) and Figure 11 show that MCF-7 cells grown in HITS medium with drug and different FCS concentrations for a total time of 78 hours (72 hours with drug plus 6 hours incubation with [methyl-³H] thymidine) gave similar results by incorporation of [methyl-³H] thymidine as for cell counting; the level of thymidine incorporation decreased (i.e. level of inhibition of incorporation increased). Furthermore, no apparent change in incorporation was evident for cells incubated in HITS medium by 8-Cl cAMP.

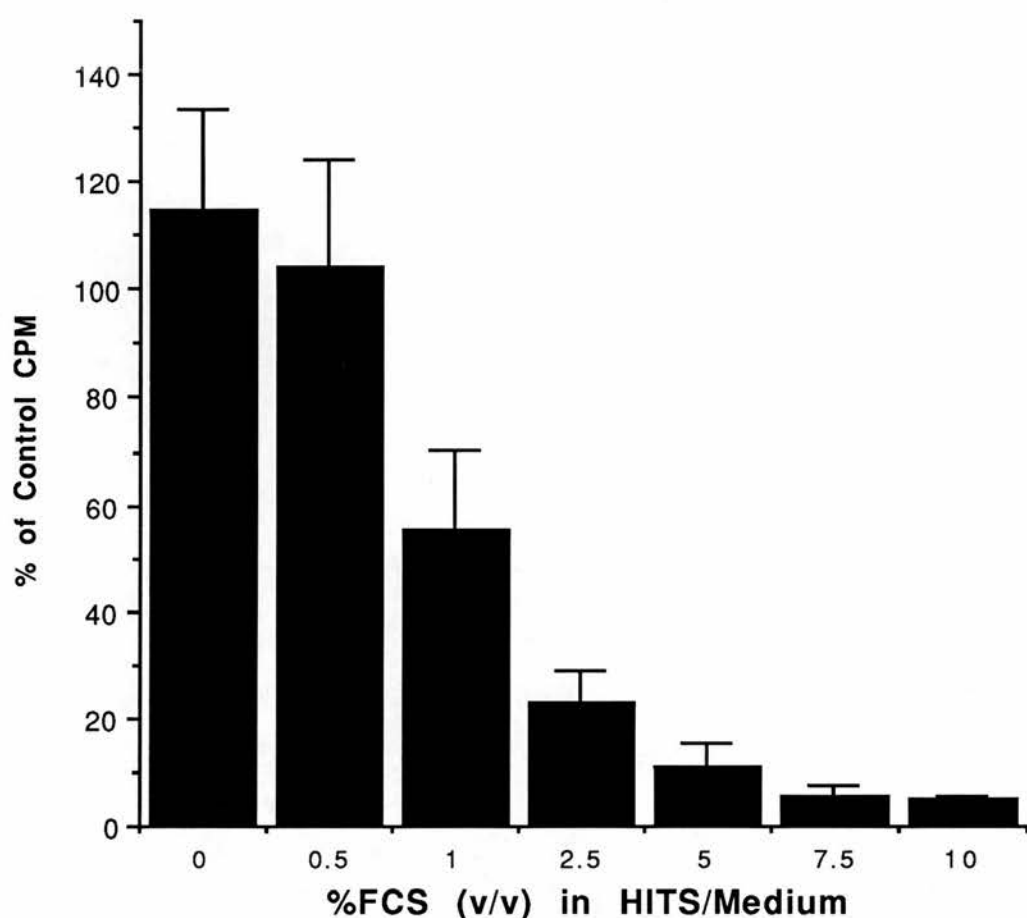


FIGURE 11:

The effect of 8-Cl cAMP (10 μ M) on the incorporation of [methyl- 3 H] thymidine by MCF-7 cells cultured for 78 hours in DMEM containing HITS and different concentrations of FCS (v/v).

Control MCF-7 cells were cultured in the absence of 8-Cl cAMP (100% incorporation).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

3.14 CONCENTRATION DEPENDENCE OF THE RESPONSE TO 8-Cl cAMP AND 8-Br cAMP

Using the thymidine uptake assay (section 2.1.6) the effect of different concentrations of 8-Cl cAMP and 8-Br cAMP on the growth inhibition of MCF-7 cells was determined. MCF-7 cells were incubated in the absence (control) or presence of a concentration range of 8-Cl cAMP (0.5 - 10 μ M) or 8-Br cAMP (30 - 360 μ M) for 72 hours, cultured in HITS medium or HITS medium containing 10% FCS (v/v). After incubation, the level of [methyl- 3 H] thymidine uptake was measured (section 2.1.6).

MCF-7 cells cultured in HITS medium did not appear to have thymidine incorporation affected by 8-Br cAMP (Figure 13). However, 8-Br cAMP did inhibit the incorporation of thymidine by MCF-7 cells, but like 8-Cl cAMP appeared to be dependent on the presence of serum. The level of inhibition increased with concentration of 8-Br cAMP and plateaued off by 250 μ M. Concentrations of less than 6 μ M 8-Cl cAMP did not appear to affect thymidine incorporation by MCF-7 cells cultured in HITS medium containing 10% FCS (v/v) (Figure 12).

8-Br cAMP is less potent at affecting proliferation compared to 8-Cl cAMP. An estimated IC_{50} derived from these plots are 5.5 μ M for 8-Cl cAMP and 50 μ M for 8-Br cAMP. This compares with the reported values of 1 μ M and 57 μ M for a five day exposure to 8-Cl cAMP and 8-Br cAMP, respectively (Katsaros *et al.*, 1988)

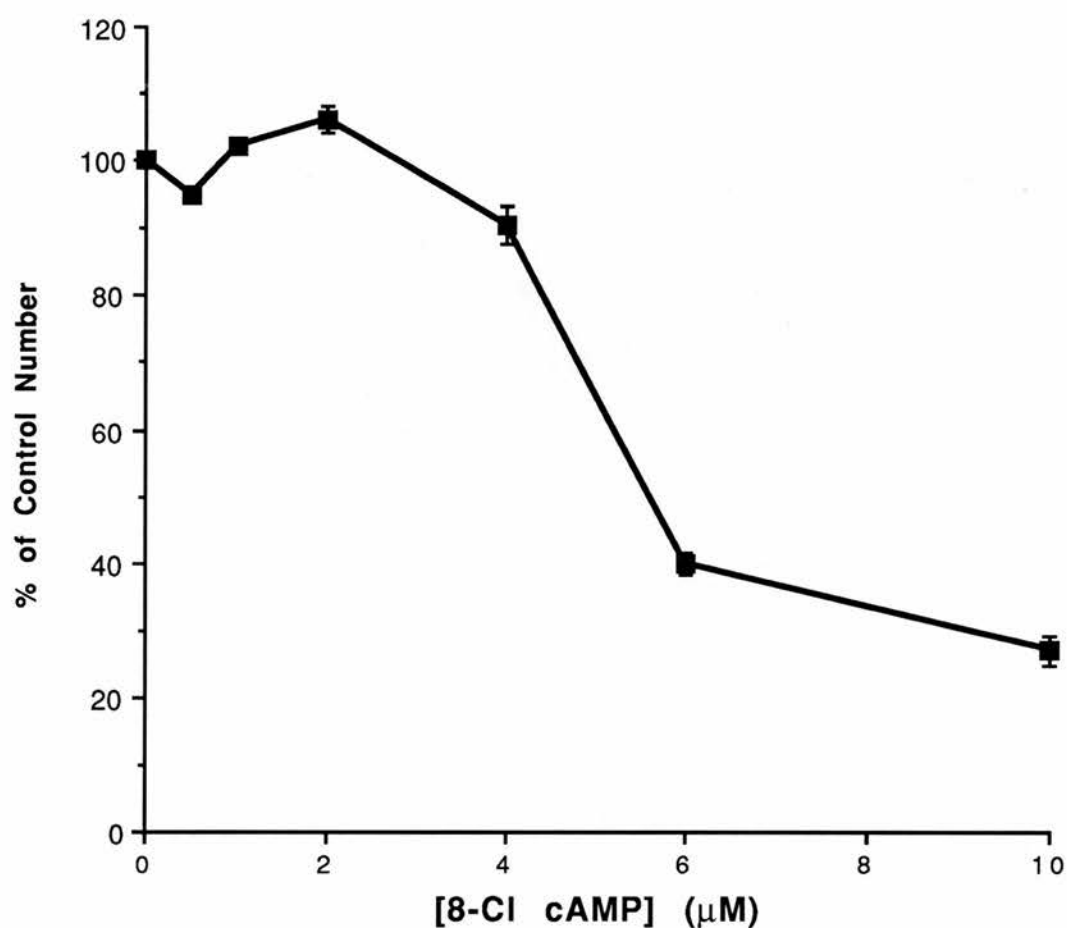


FIGURE 12:

The effect of different concentrations of 8-Cl cAMP on the incorporation of [methyl- ^3H] thymidine in MCF-7 cells cultured for 78 hours in HITS medium containing 10% FCS (v/v).

Control cells were cultured in the absence of drug (100% incorporation).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

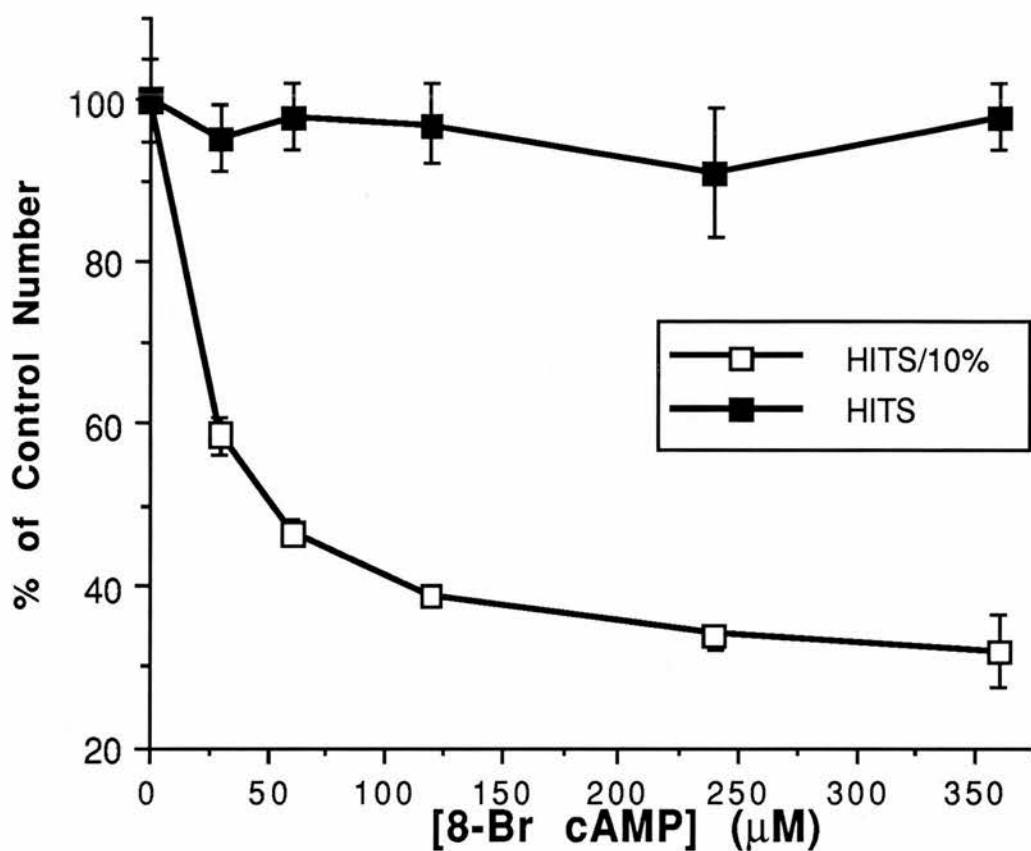


FIGURE 13:

The effect of different concentrations of 8-Br cAMP on the incorporation of [methyl-³H] thymidine in MCF-7 cells cultured for 78 hours in HITS medium (**HITS**) or HITS medium containing 10% FCS (v/v) (**HITS/10%**).

Control cells were cultured in the absence of drug (100% incorporation).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

3.15 HEAT INACTIVATION OF THE SERUM-DEPENDENT 8-Cl cAMP INHIBITORY RESPONSE

3.15.1 Heat Lability Of A Putative Serum Factor (IF-s):

To determine the nature of the factor(s) (IF-s) responsible for this serum-dependent 8-Cl cAMP response, aliquots of FCS were heated for varying periods of time and at different temperatures. FCS (500 μ l) was heated to 97 °C using a microfuge tube heating block for a period of 1, 3, 10 or 30 minutes then cooled on ice. This serum was added to HITS medium to give a 10% (v/v) solution and assayed for 8-Cl cAMP dependent growth inhibition with by thymidine uptake (section 2.1.6). The control for this experiment was media added to cells without the addition of drug.

The result shown in Figure 14 demonstrate that FCS heat treated for 1 minute at 97 °C still retained the same level of activity as FCS not heat treated. However, FCS heated for 3 minutes or more did not retain IF-s activity.

3.15.2 Temperature Of Heat Inactivation:

To determine the temperature at which IF-s was heat inactivated, FCS (500 μ l) was heat treated for 15 minutes at different temperatures ranging from 60 °C to 95 °C with 5 °C increments. The serum was then assayed as described in section 3.15.1.

All growth inhibition (IF-s activity) was lost at these temperatures except with the untreated FCS (data not shown).

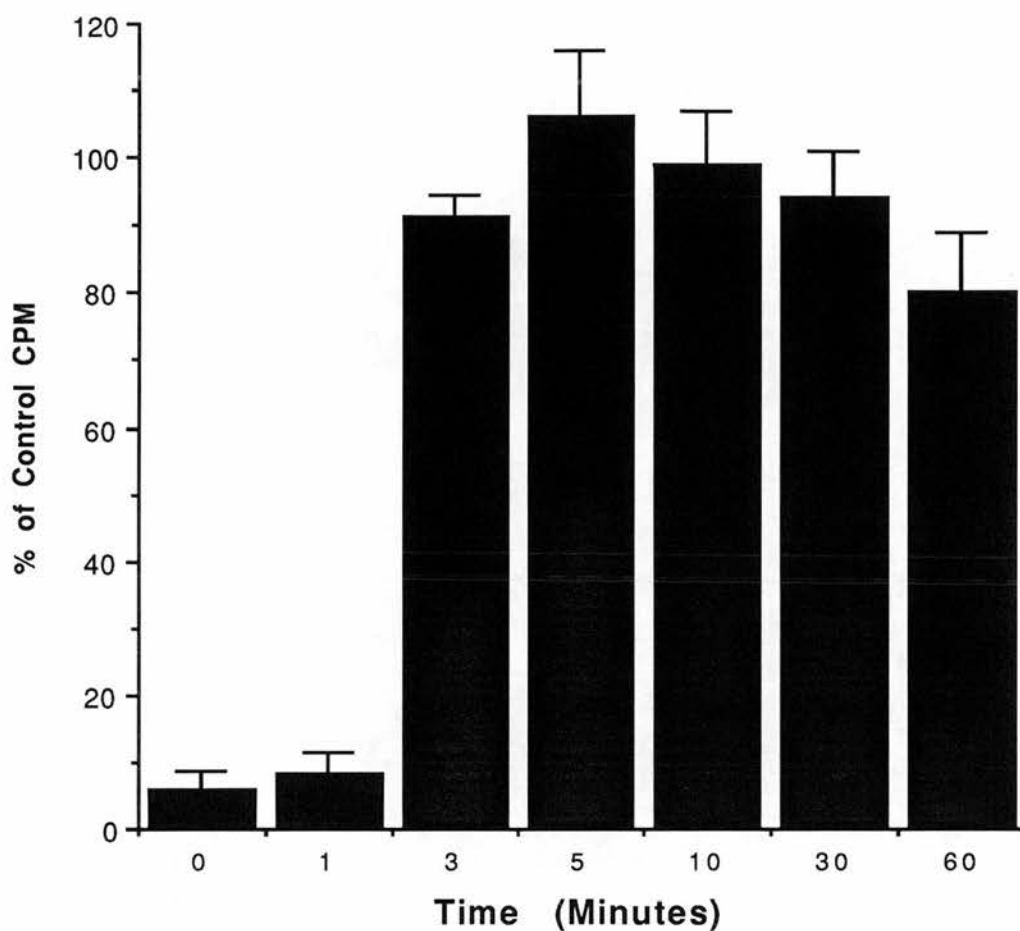


FIGURE 14:

The effect of 8-Cl cAMP (10 μ M) on the incorporation of [methyl- 3 H] thymidine by MCF-7 cells cultured for 78 hours in HITS medium containing 10% FCS (v/v) heat treated at 97 $^{\circ}$ C for varying periods of time.

Control MCF-7 cells were cultured in the absence of drug (100% incorporation).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

3.16 MOLECULAR WEIGHT ESTIMATION OF IF-s USING MOLECULAR WEIGHT CUT OFF FILTERS

3.16.1 Introduction:

To isolate IF-s from serum and determine a rough estimation of its molecular weight, a range of Centricon™ filters were utilised to produce fractions containing different molecular weights. Centricon™ filters are composed of specialised membrane with pore sizes that allow passage of certain molecular weight species. These filters are supplied in a range of molecular weight cut offs including 10 kDa, 30 kDa and 100 kDa.

3.16.2 Molecular Weight Estimation Of IF-s:

A sample of FCS (2 ml) was applied to the top of the filters and centrifuged at 4 °C (see section 2.11.1 for centrifugal force for the different filters). Molecular weight species too large to pass through the membrane concentrate above the filter while the smaller molecules and water pass through the filter (filtrate). The fractionation was completed when the volume of the concentrate did not reduce further. Both fractions were collected and the volume of the concentrate was adjusted to the original volume applied to the column with PBS. The two fractions were then added individually and in combination to HITS medium to give a concentration of 10% (v/v). The media was then filter sterilised with 0.22 µm filter units before being measured for IF-s activity with the thymidine uptake assay (section 2.1.6). A volume of the original serum applied to the filters was retained and filter sterilised to be used for a positive IF-s activity control. In addition, a volume of the fractions were run on non-denaturing 7.5% PAGE gels to check the protein molecular weight

of each fraction.

Figure 15 shows the data for FCS fractionated using the 100 kDa filter. The concentrate (fraction retained above the filter) contained the same level of IF-s activity as for unfiltered FCS. FCS fractionated with the 10 kDa and 30 kDa filters produced a similar result; the concentrate retained IF-s activity (data not shown). The filtrate did not appear to retain any activity in the presence of 8-Cl cAMP (10 μ M).

These data suggest that IF-s has a rough molecular weight of greater than 100 kDa. However, it is not possible to conclude this since the Coomassie blue stained PAGE gel (plate 8) of the fractions from the 100 kDa unit suggest the fidelity of the cut off filter was very poor. The concentrate appears to contain a high concentration of a 67 kDa protein compared to the filtrate. Nevertheless, these filters do provide a very convenient method for concentrating FCS and retaining the IF-s within the concentrate.

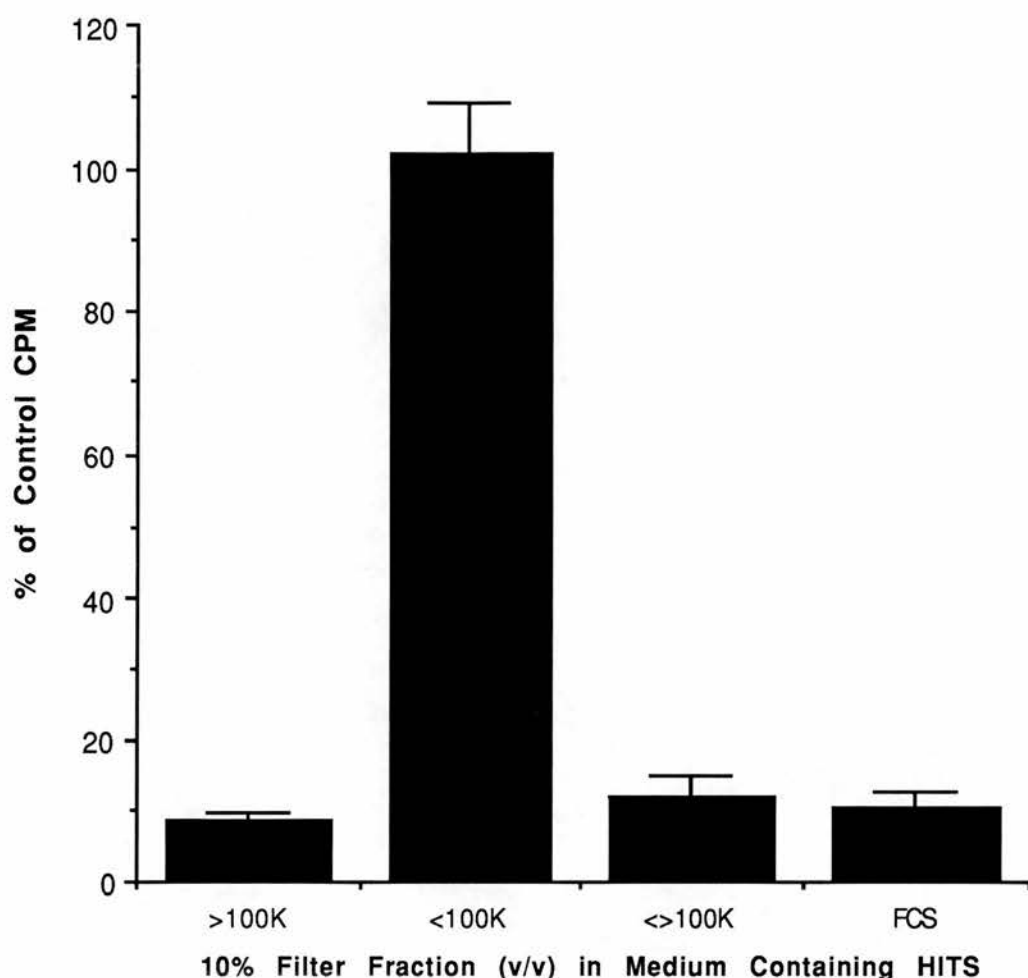


FIGURE 15:

The effect of 8-Cl cAMP (10 μ M) on the incorporation of [methyl- 3 H] thymidine in MCF-7 cells cultured for 78 hours in HITS medium containing 10% (v/v) FCS fraction from a 100 kDa Centricon filter.

Control MCF-7 cells were not treated with drug (100% incorporation).

>100K : concentrate; did not pass through filter.

<100K : filtrate; passed through filter.

<>100K : both concentrate and filtrate added at 10% (v/v) each.

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

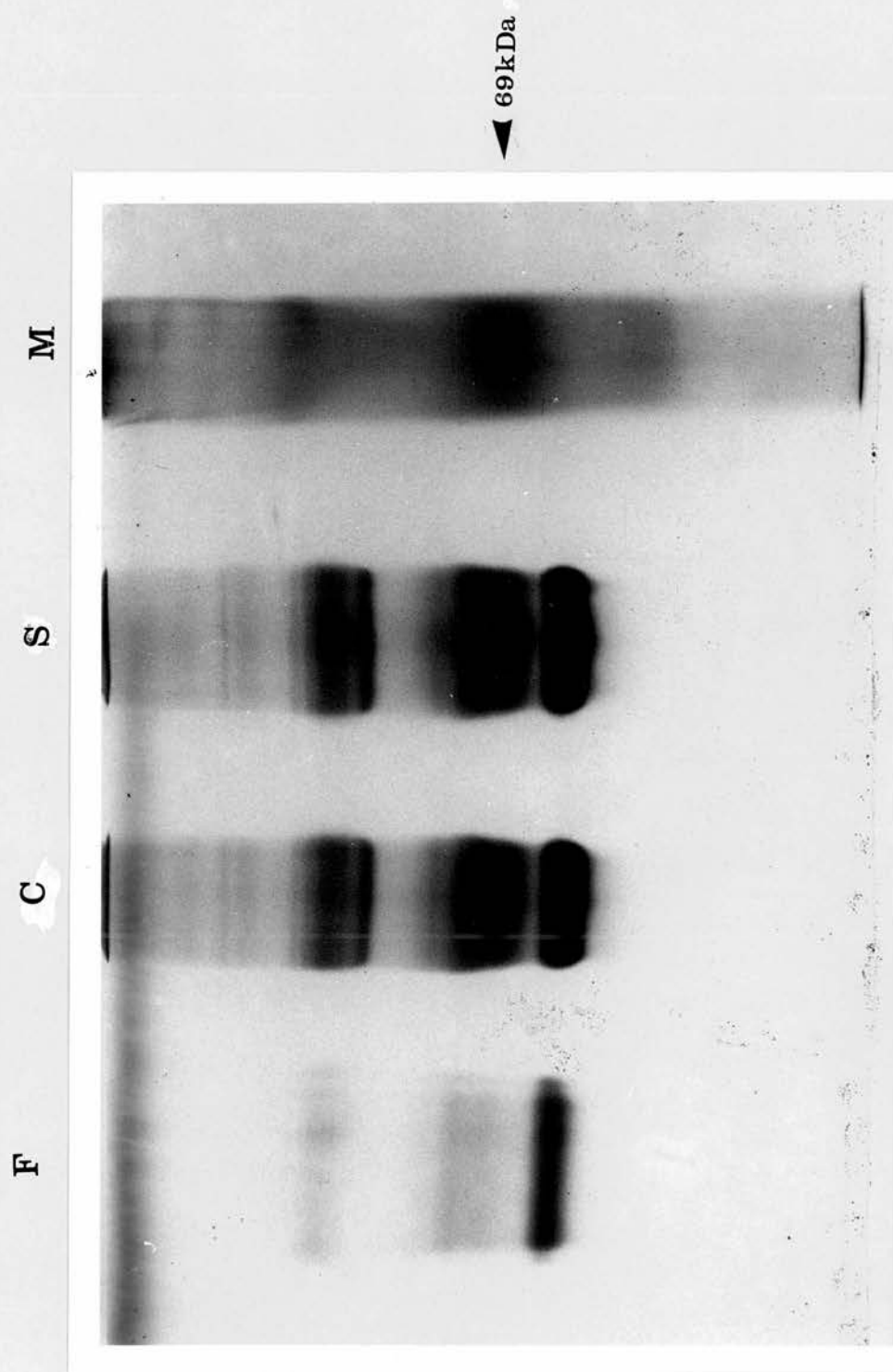


Plate 8:

Coomassie blue stained non-denaturing 7.5% PAGE gel of foetal calf serum filtered with a 100 kDa Centicon filter.

S- FCS; **C-** concentrate; **F-** filtrate; **M-** marker proteins.

Each lane was loaded with 10 μ l.

3.17 GEL FILTRATION CHROMATOGRAPHY OF CONCENTRATED FCS

3.17.1 Introduction:

Small micron sized beads composed of cross-linked polysaccharide or polyacrylamide can be used to separate a mixture of proteins; this technique is known as gel filtration chromatography. These beads have a fine mesh that allows the movement of small proteins through them and larger proteins have a greater resistance to enter these spaces. Proteins can be separated based on this principle with the larger proteins being eluted from a column composed of these beads first followed by the smaller molecular weight species.

3.17.2 10 cm x 7 mm Column:

A volume of FCS was concentrated to a fifth of its original volume using a 10 kDa Centricon filter (section 2.11.1). The concentrate (2 ml) was applied to an S-200 sephacryl column (10 cm x 7 mm diameter), pre-equilibrated with PBS, and 750 μ l fractions collected from the column (section 2.11.2). A total of twenty fractions were collected. Protein concentration for each eluted fraction was estimated by spectrophotometric absorbance at 280 nm. Each fraction was added to HITS medium to give a concentration of 10% (v/v) and filter sterilised (0.22 μ m units) before being assayed with MCF-7 cells for IF-s activity by thymidine uptake (section 2.1.6). A volume of the fractions were resolved with a 7.5% SDS-PAGE gel to visualise ^{the} to proteins (Plate 9).

Figure 16 displays the protein concentration of fraction and the inhibitory activity by 8-Cl cAMP (10 μ M). Fractions 10 and 11 contain the highest protein concentration and is probably due to serum albumin (approximate molecular weight of 67 kDa), since it is the most abundant protein in serum. The majority of the 8-Cl cAMP growth inhibition coincided with fractions 6 and 7, and then diminished to give no activity by fraction 13.

These fractions probably contain the majority of the unknown factor IF-s. Plate 9 shows that fraction 6 and 7 does not have as much of the 67 kDa protein as the later fractions, suggesting that IF-s is not albumin.

3.17.3 11 cm x 18 mm Column:

A second S-200 sephacryl column with a larger volume (11 cm x 18 mm diameter) was run to allow a larger volume of concentrate (3 ml) to be fractionated. A total of forty, 1.5 ml fractions were collected and protein concentration and IF-s activity measured as described for section 3.17.2.

This column gave a more detailed protein profile for FCS (Figure 17) compared to the smaller volume column. The majority of the 8-Cl cAMP growth inhibitory effect was contained in fractions 10, 11 and 12. Again, the majority of the activity was eluted before the major protein peak.

Criticism of Gel Filtration Chromatography Experiments (section 3.17):

Ideally, a sample volume of 0.5 - 5 % of the column bed volume should be applied for good resolution of sample components (Gel Filtration in Theory and Practice, *Pharmacia Fine Chemicals*). The two columns used in section 3.17 had volumes of 3.85 cm³ (100 mm x 7 mm diameter), and 5.53 cm³ (110 mm x 18 mm diameter), with sample volumes of 2 ml and 3 ml, respectively. For good resolution with these columns the maximum sample volumes of 0.2 ml and 0.28 ml should have been applied. The sample volumes applied to these columns (section 3.17) were approximately 10 times greater than the volume required for good resolution, and therefore inappropriate for good resolution of serum components by these gel filtration columns. A smaller sample volume may have improved separation of serum components, however the IF-s separated into fractions may not have been concentrated enough to detect a biological response. would!

Gel filtration of serum in section 3.17 did enable crude extraction of IF-s activity into a small number of fractions, eliminating a large proportion of other serum components, such as albumin. These fractions could then have been used for more detailed gel filtration chromatography to study the protein profile of the active fractions containing IF-s.

the gel illustrated (Plate 9)
gives no support to your contention
that 'elimination' of albumin
from any of your samples was successfully
achieved.

post-column
concentration?

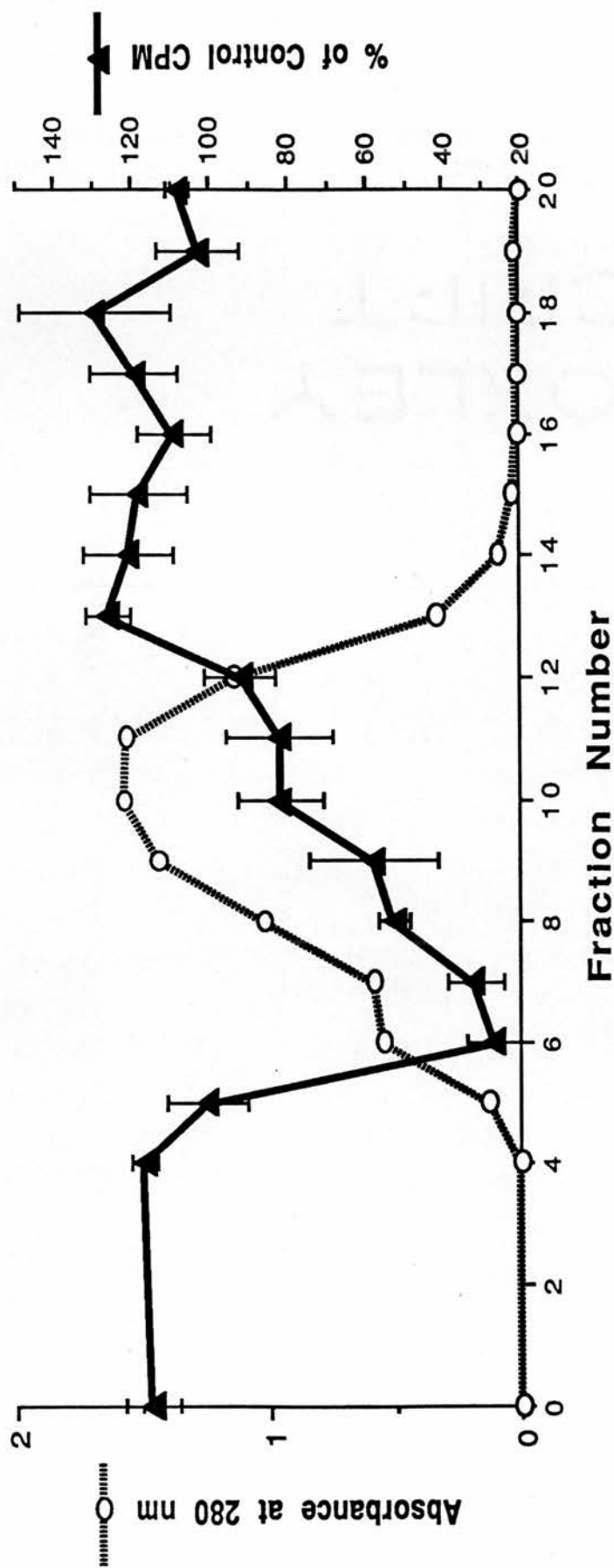


FIGURE 16:

The effect of 8-Cl cAMP (10 μ M) on the incorporation of [methyl-3H] thymidine by MCF-7 cells cultured for 78 hours in HITS containing 10% (v/v) of a FCS fraction (see section 3.17.2).

Fractions were obtained from an S-200 column: dimensions 100 mm x 7 mm diameter.

Control cells were cultured in the absence of 8-Cl cAMP (i.e. 100%).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

Protein content determined from absorbance of sample at 280 nm.

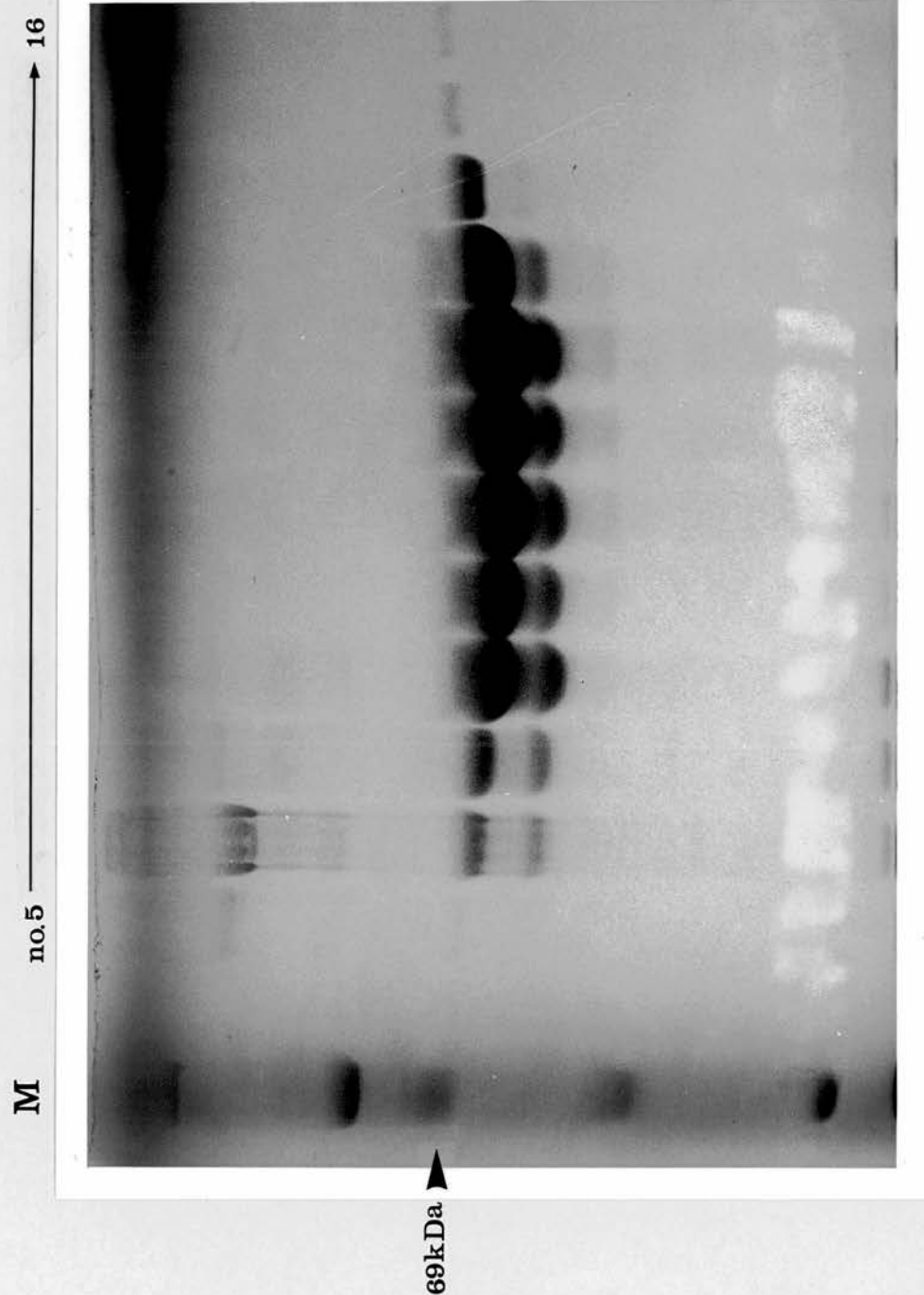


Plate 9:

Coomassie blue stained 7.5% SDS-PAGE gel of FCS fractions collected from a 10 cm x 7 mm diameter S-200 sephacryl column.

Samples were load with a volume of 5 μ l; **M**- marker proteins (10 μ l).

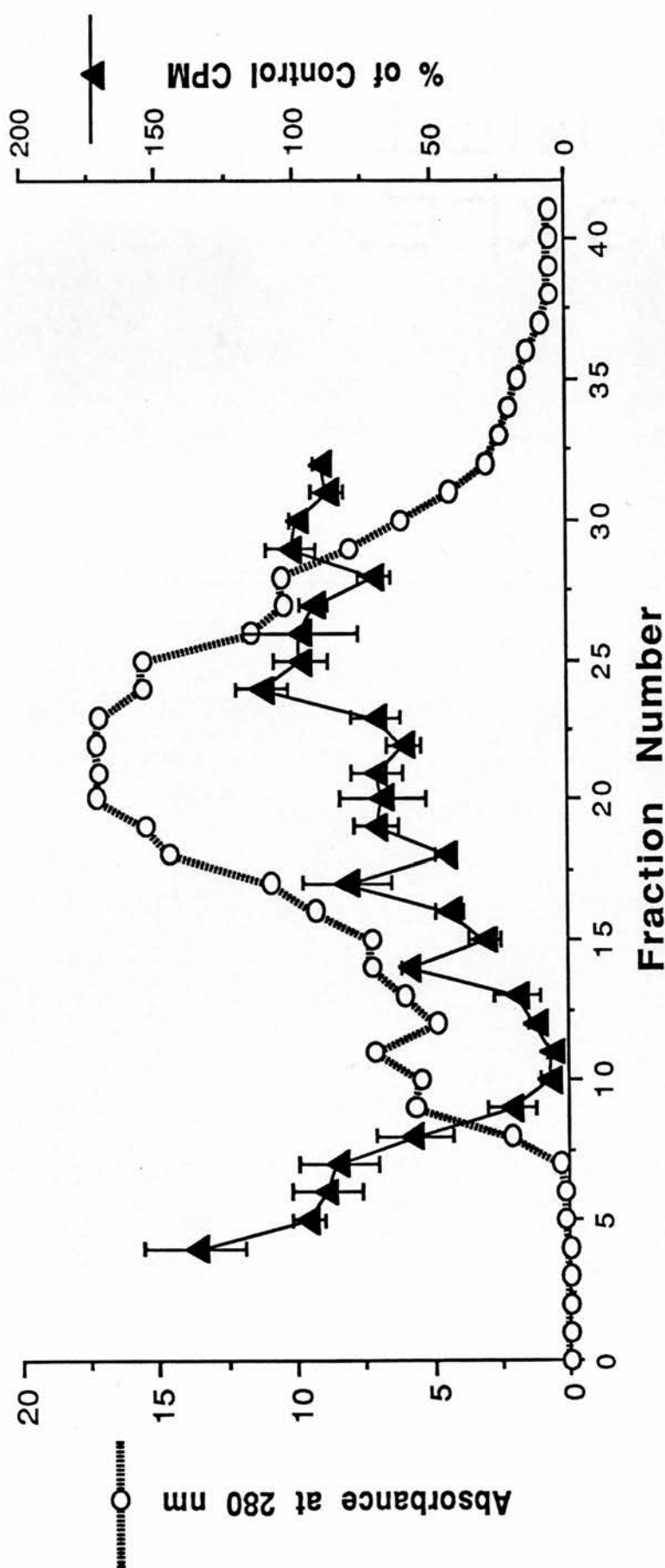


FIGURE 17:

The effect of 8-Cl cAMP (10 μ M) on the incorporation of [methyl-3H] thymidine by MCF-7 cells cultured for 78 hours in HITS medium containing 10% (v/v) of a FCS fraction (see section 3.17.3).

Fractions were obtained from an S-200 column: dimensions 110 mm x 18 mm diameter.

Control cells were cultured in the absence of 8-Cl cAMP (i.e. 100%).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

Protein content determined from absorbance of sample at 280 nm.

3.18 THE PRESENCE OF IF-s IS NOT REQUIRED DURING THE PERIOD OF CELL CULTURE FOR INHIBITION BY 8-Cl cAMP

3.18.1 Introduction:

The mechanism by which IF-s confers sensitivity of MCF-7 cells to 8-Cl cAMP is not known. IF-s could be acting directly on the cell making it sensitive to 8-Cl cAMP, alternatively IF-s could be combining with 8-Cl cAMP to form an active complex or IF-s could modify the drug into an active form. As previously demonstrated in section 3.16, IF-s is unable to pass through the 10 kDa filter unit. In view of this, if IF-s combines to 8-Cl cAMP then the complex would not pass through the filter and the filtrate would not show any activity. However, if IF-s activates 8-Cl cAMP then this might be able to pass through the filter unit and the filtrate would contain an antiproliferative effect even though IF-s is not present.

3.18.2 IF-s Activates 8-Cl cAMP:

To determine if IF-s activates 8-Cl cAMP, HITS medium containing 10% FCS (v/v) and 8-Cl cAMP (10 μ M) (3 ml) was incubated for varying time periods at 37 °C. At each time point the medium was centrifuged through a 10 kDa filter (section 2.11.1) and the filtrate collected for assaying for inhibitory activity by thymidine uptake (section 2.1.6). The control for this particular experiment was a volume of HITS medium containing 10% FCS (v/v) incubated for 72 hours in the absence of drug. A sample with 8-Cl cAMP was also incubated for 72 hours at 4 °C.

The data in Figure 18 is expressed as a percentage of the incorporation data for filtered medium incubated for 72 hours in the absence of 8-Cl cAMP. The results demonstrate that the growth inhibitory activity can pass through the filter, and incubation time increased the level of inhibition of [methyl-³H] thymidine incorporation. The medium with drug incubated at 4 °C for 72 hours had a level of inhibition equivalent to medium incubated for 6-12 hours at 37 °C. Addition of 8-Cl cAMP (10 μM) to the time point zero medium did not produce an inhibitory activity, suggesting that IF-s was removed by filtration (Figure 18). Addition of 8-Cl cAMP (10 μM) to the other filtrate solutions also showed no further effect on thymidine incorporation.

These data suggest that IF-s was not required to interact directly with the cell to sensitize it towards 8-Cl cAMP, since filtration with a 10 kDa filter removed the factor. Moreover, the data implies that IF-s does not complex to 8-Cl cAMP since this would not have passed through the filter. It is possible that IF-s activates 8-Cl cAMP conferring growth inhibitory properties to the drug. This activation appeared to be time dependent and temperature sensitive.

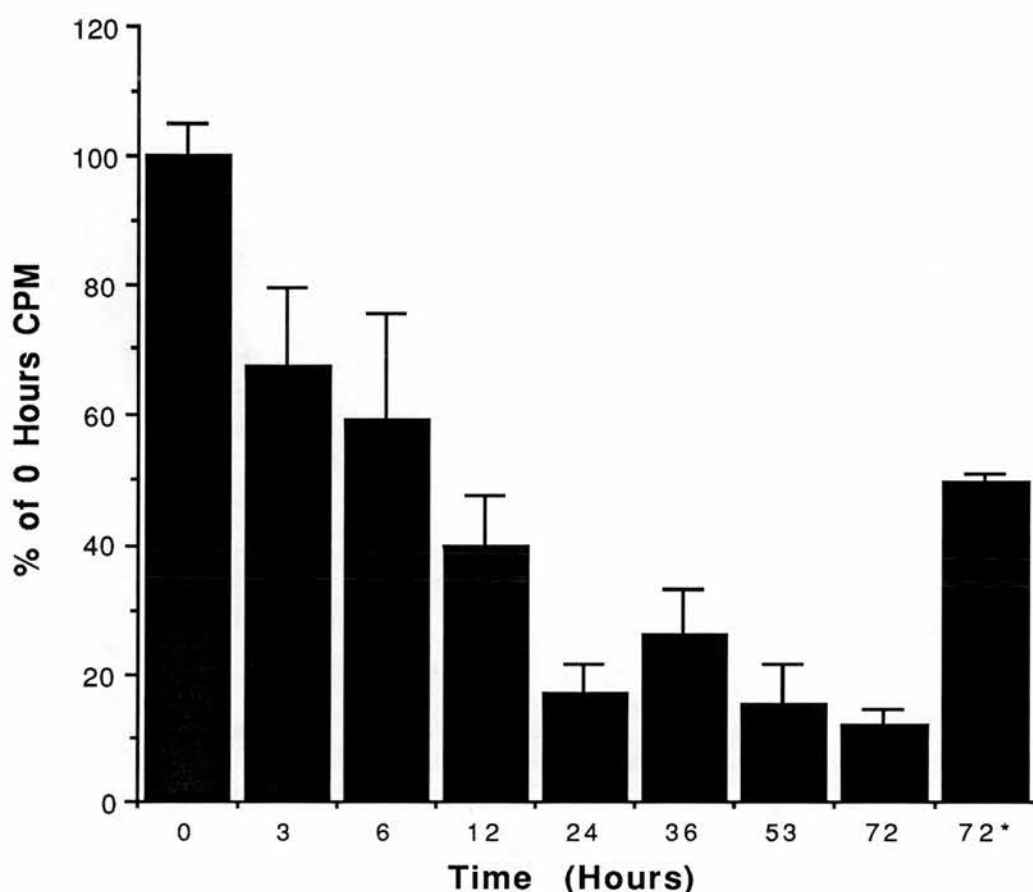


FIGURE 18:

Production of an active, filterable 8-Cl cAMP derivative by preincubation in serum containing medium.

HITS medium containing 10% FCS (v/v) and 8-Cl cAMP (10 μ M) was preincubated at 37 $^{\circ}$ C for increasing periods of time before filtering through a 10 kDa Centricon filter. The effect of this medium was assayed by the incorporation of [methyl- 3 H] thymidine with MCF-7 cells cultured for 78 hours. Control MCF-7 cells were cultured in HITS medium containing 10% FCS (v/v) preincubated for 72 hours without drug before filtering (100% incorporation).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

* medium incubated at 4 $^{\circ}$ C for 72 hours.

3.19 EFFECT OF IBMX ON 8-Cl cAMP INHIBITION OF MCF-7 CELLS CULTURED IN HITS MEDIUM CONTAINING 10% FCS (V/V)

3.19.1 Introduction:

According to a recent report by Van Lookeren Campagne *et al.* (1991), endogenous phosphodiesterases (PDE) and 5'-nucleotidases in serum can metabolise 8-Cl cAMP, in the absence of cells, to form a toxic 8-Cl adenosine metabolite which was responsible for the antiproliferative effect in their culture system. This metabolism was demonstrated with 100 μ M of drug. It has been suggested however that a 10 μ M concentration of 8-Cl cAMP is below the concentration required to activate phosphodiesterases (Cho-Chung, 1989).

3.19.2 Isobutylmethylxanthine (IBMX):

To investigate the involvement of phosphodiesterases in the growth inhibitory response of 8-Cl cAMP, the general phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) was added to culture medium. A stock solution of IBMX was dissolved at a concentration of 5 mM in medium containing HITS. From this IBMX was added to HITS medium containing 10% FCS (v/v) to give a range of concentrations from 0.025 - 0.5 mM and assayed in the presence of 8-Cl cAMP (10 μ M) by thymidine uptake (section 2.1.6). The controls for this experiment contained IBMX with no 8-Cl cAMP.

IBMX in medium not containing 8-Cl cAMP appeared to inhibit thymidine uptake; for example 0.025 mM produced 27% inhibition and 75% with 0.5 mM. However, in the presence of 8-Cl cAMP

(10 μ M), all concentrations of IBMX used dramatically reduced the inhibitory activity of the analogue (Figure 19). For example, in the absence of IBMX, 8-Cl cAMP inhibited growth by 81% of the control and in the presence of 0.025 mM IBMX growth was only inhibited by 25% of the control.

Even though increasing concentrations of IBMX inhibited cellular proliferation, in the presence of 8-Cl cAMP (10 μ M) growth inhibition was only approximately 25%. These data suggest that PDE may be involved in the serum-dependent 8-Cl cAMP growth inhibition of cells.

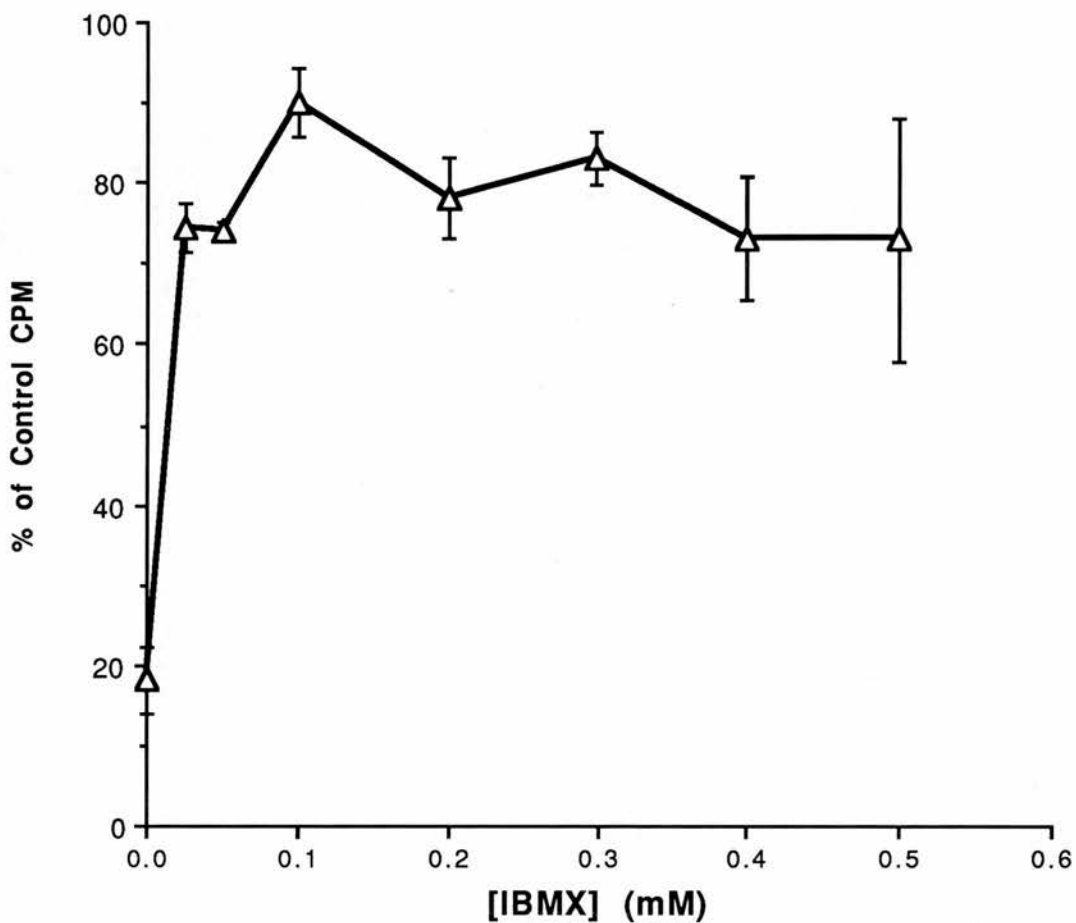


FIGURE 19:

The effect of isobutylmethylxanthine (IBMX) on the incorporation of [methyl-³H] thymidine with MCF-7 cells cultured for 78 hours in HITS medium containing 10% FCS (v/v) plus 8-Cl cAMP (10 μ M).

Control cells were cultured in the absence of 8-Cl cAMP.

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

3.20 EFFECT OF PDE ON MCF-7 CELLS CULTURED IN HITS MEDIUM WITH 8-Cl cAMP

3.20.1 Introduction:

The data from section 3.19 suggest that PDE may be involved in the 8-Cl cAMP growth inhibition. If PDE is responsible for the 8-Cl cAMP antiproliferative effect then addition of these enzymes to HITS medium with drug should confer growth inhibition of MCF-7 cells.

3.20.2 Phosphodiesterases:

A crude preparation of PDE from bovine heart (1 unit will hydrolyse 1.0 μ M of cAMP to AMP per minute at pH 7.5 at 30 °C) was dissolved in HITS medium at a concentration of 0.01 - 0.10 units per ml and assayed with MCF-7 cells for thymidine uptake (section 2.1.6) in the presence or absence (controls) of 8-Cl cAMP (10 μ M).

In the presence of 0.01 units/ml of PDE, 8-Cl cAMP inhibited proliferation of MCF-7 cells by 95% relative to control (no drug; 100%). A further increase in the level of growth inhibition was not evident with higher concentrations of PDE (Figure 20).

The inhibition with 0.01 units of PDE per ml was more dramatic compared to previous observations with HITS medium containing 10% FCS (v/v); approximately 80% inhibition. PDE catalyse the conversion of cAMP to AMP; this observed growth inhibition may be due to 8-Cl AMP. However, the PDE was a crude extract and may contain other enzymes which may metabolise 8-Cl AMP to other products including 8-Cl adenosine.

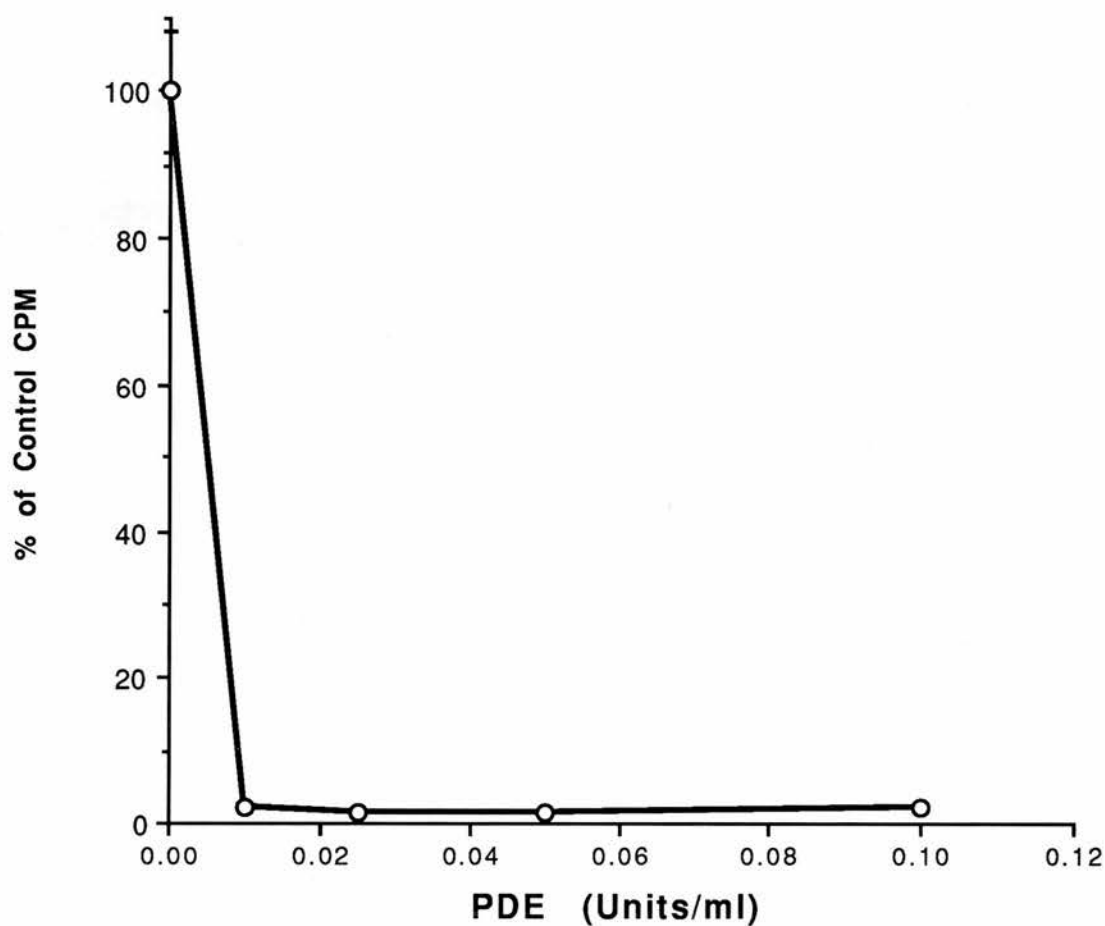


FIGURE 20:

The effect of cAMP-dependent phosphodiesterases (PDE) on the incorporation of [methyl-³H] thymidine by MCF-7 cells cultured in HITS medium containing 10% FCS (v/v) plus 8-Cl cAMP (10 μ M) for 78 hours.

Control cells were cultured in the absence of 8-Cl cAMP.

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

3.21 HPLC ANALYSIS FOR 8-Cl cAMP METABOLITES

3.21.1 Introduction:

Data from sections 3.19 and 3.20 showed that IBMX can partially block 8-Cl cAMP induced inhibition of growth in serum containing medium, and PDE can confer an 8-Cl cAMP response in HITS medium. Together these results implicate the involvement of PDE in the mechanism of 8-Cl cAMP inhibition of cellular proliferation. To determine if serum PDE metabolise 8-Cl cAMP in culture the HPLC method described by Van Lookeren Campagne *et al.* (1991) was employed (section 2.11.4).

3.21.2 Calibration:

Standards of 8-Cl cAMP and 8-Cl adenosine were dissolved in distilled water and used to identify the retention times of the drugs to the column and optimise conditions to produce good resolution of the peaks. Each standard produced a distinct peak by HPLC analysis suggesting there was little or no contaminating products (Chromatogram 1). A flow rate of 1.4 ml/minute and a 30% methanol buffer (v/v) was eventually used to optimise the system. Calibration plots were constructed from known concentrations of standard 8-Cl cAMP and 8-Cl adenosine.

The peak areas for known concentrations of standard were plotted to produce a calibration plot (Figure 21). The peak areas detected appear to relate to the concentration of standard applied to the column. The gradient of the line for both plots are very similar, implying that equimolar concentration of both compounds give equivalent peak areas. Standards were also dissolved in HITS medium and produced identical plots compared to standard

dissolved in water. Furthermore, no loss was found in peak area for a concentration of standard centrifuged through a 10 kDa filter unit, demonstrating that these compound are free to pass through the filter.

3.21.3 Metabolism Of 8-Cl cAMP By FCS:

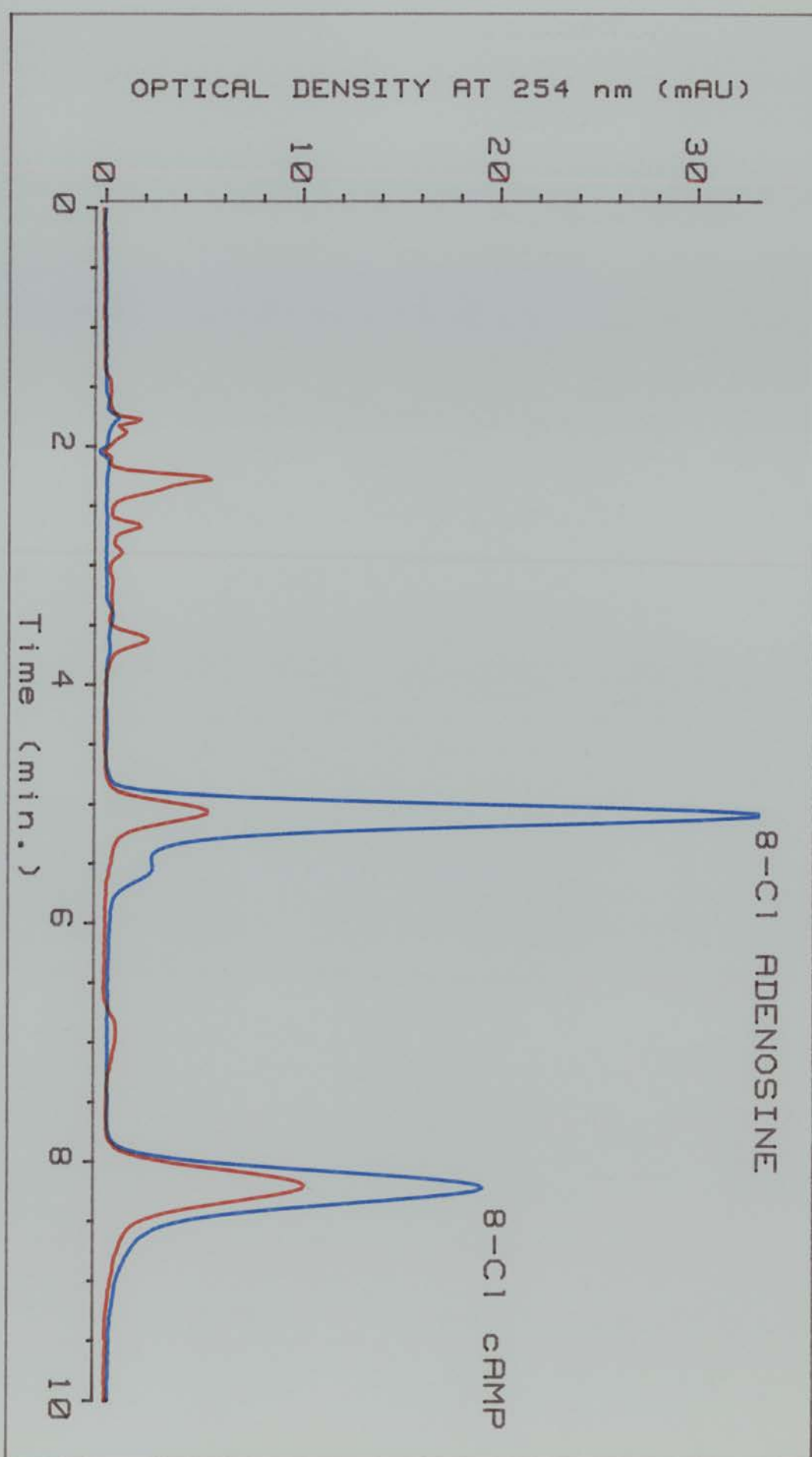
To investigate if 8-Cl cAMP was metabolised by FCS, HITS medium containing different concentrations of FCS was incubated at 37 °C with either 100 µM or 10 µM 8-Cl cAMP for 72 hours. The media was centrifuged through a 10 kDa Centricon filter to remove the majority of the FCS proteins, and the filtrate diluted 1 : 20 with running buffer (section 2.11.3) before analysis by HPLC (section 2.11.4). Standard calibration plots were also constructed.

Chromatogram 1 shows an overlay plot of two samples: 200 µM of 8-Cl cAMP and 8-Cl adenosine standard dissolved in water; and a 100 µM 8-Cl cAMP sample incubated for 72 hours in HITS medium containing 10% FCS (v/v). The peaks eluted from the column before four minutes were also present in HITS medium alone. The only peak, apart from 8-Cl cAMP, that appeared after incubating drug in serum containing medium, corresponded to the 8-Cl adenosine standard (i.e. identical retention times on the column). The concentration of the 8-Cl adenosine associated peaks is plotted in Figure 22 against the concentration of serum. The concentration of the 8-Cl adenosine associated peak in medium appears to increase with respect to the serum content with both starting concentrations of 8-Cl cAMP. No metabolite peak was detected with either concentration of 8-Cl cAMP incubated in HITS medium.

This data confirms the report by Van Lookeren Campagne *et al.* (1991) that a metabolite of 8-Cl cAMP (100 μM) is produced in medium containing serum. However, this data also demonstrates that 10 μM 8-Cl cAMP is also metabolised in serum conditions. These data suggest that for previous experiments with MCF-7 cells cultured in HITS medium containing 10% FCS (v/v) and 8-Cl cAMP (10 μM), a metabolite, possibly 8-Cl adenosine was metabolised to a concentration of approximately 8 μM after a 3 day incubation. Furthermore, the concentrations of detectable metabolite found in medium with different amounts of serum appear to correlate with data that demonstrate the level of growth inhibition by 8-Cl cAMP to be dependent on serum concentration (section 3.11.4)

Chromatogram 1:

A representative chromatography trace of 8-Cl cAMP and 8-Cl adenosine standards (200 μ M) (Blue trace) overlaid with a plot for a sample of HITS medium containing 10% FCS (v/v) plus 8-Cl cAMP (100 μ M) incubated at 37 °C for 72 hours (Red trace) (see section 3.21.3).



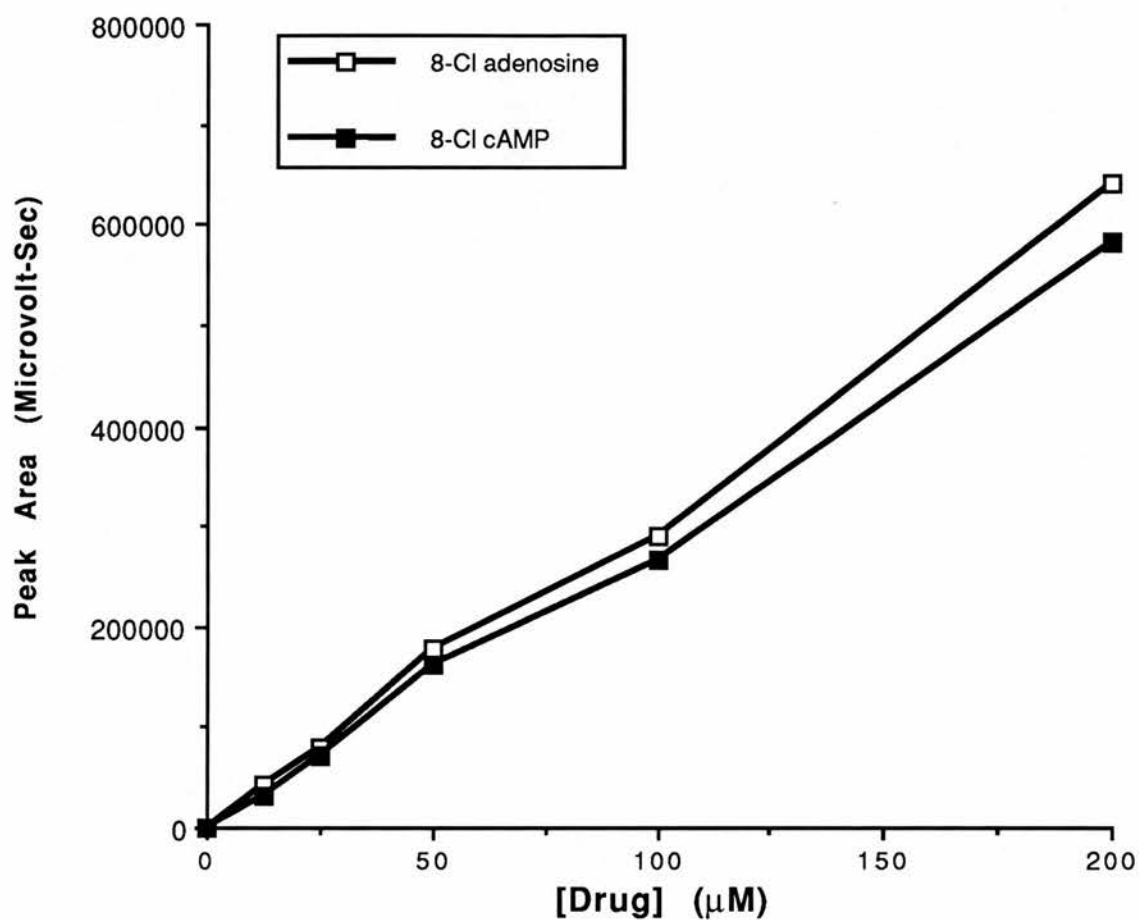


FIGURE 21:

Peak areas detected by HPLC for known concentrations of 8-Cl cAMP and 8-Cl adenosine in H_2O (see section 3.21.2).

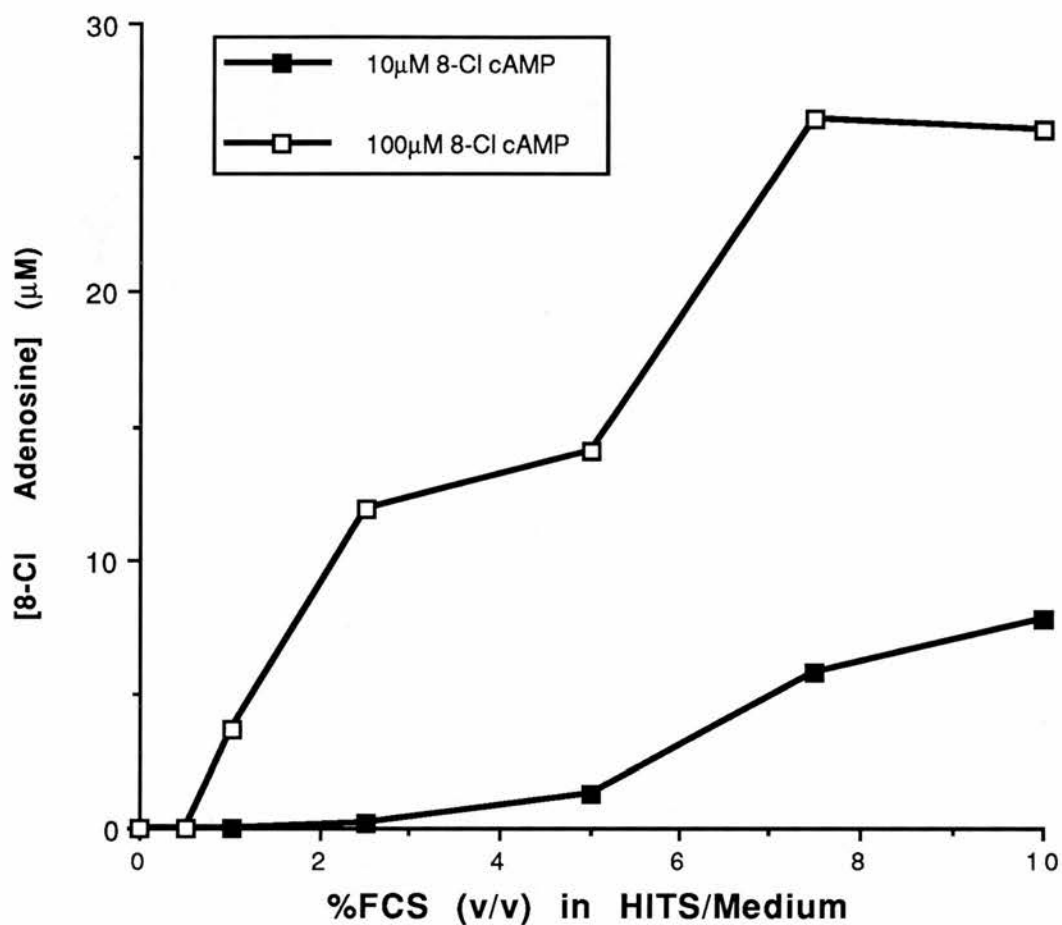


FIGURE 22:

The concentration of an 8-Cl adenosine associated metabolite peak (see Chromatogram 1) detected by HPLC analysis in HITS medium containing 10% FCS (v/v) plus 8-Cl cAMP incubated at 37 °C for 72 hours.

Medium was filtered through a 10 kDa Centricon filter before analysis by HPLC.

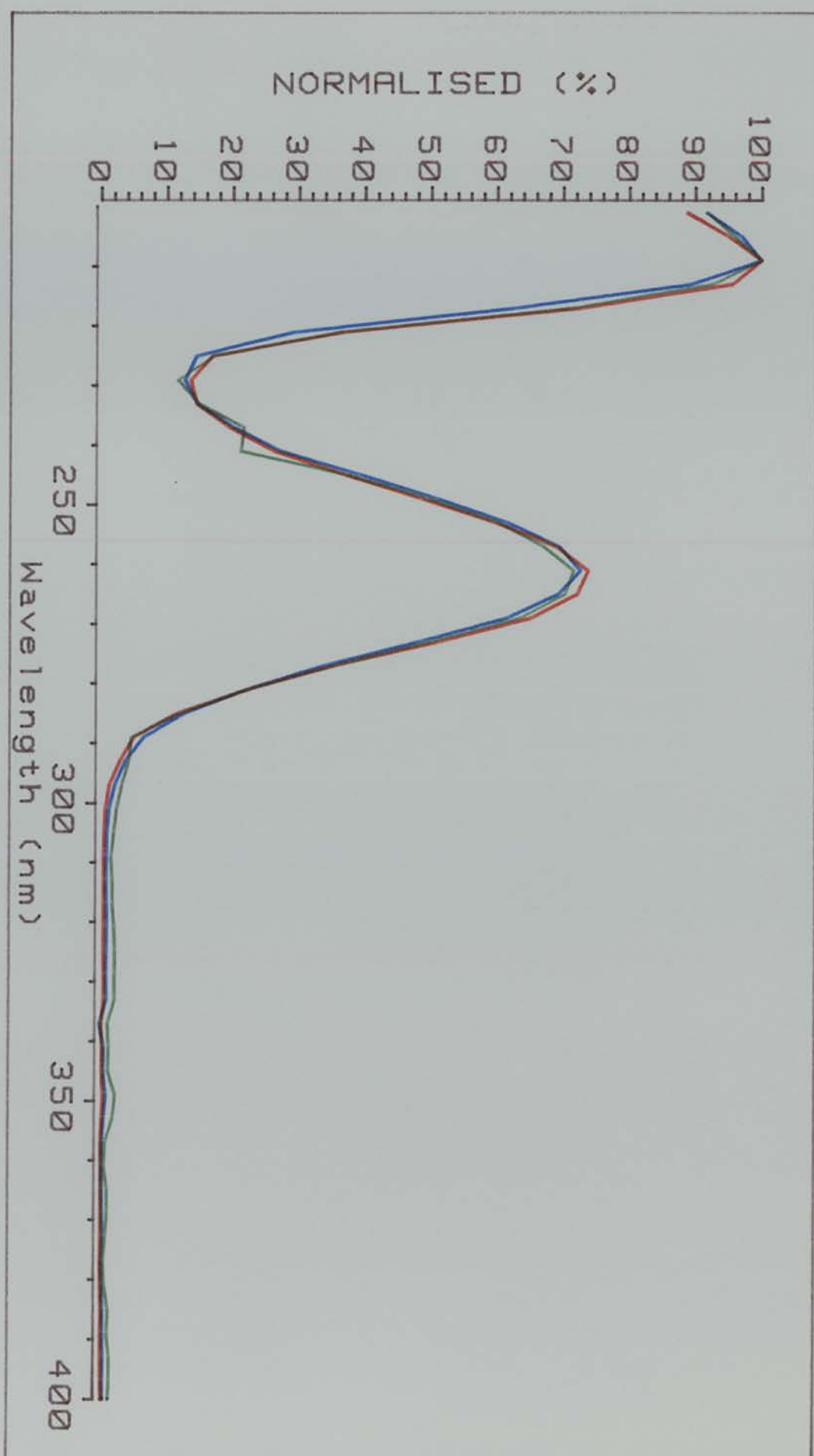
3.22 CONFIRMATION THAT THE METABOLITE PEAK IS **8-Cl ADENOSINE**

To confirm that the 8-Cl adenosine associated metabolite peak from section 3.21.3 was related to 8-Cl adenosine the metabolite peak and standards were spectrally analyzed with the HP1090 (**Hewlett Packard**). This HPLC equipment can monitor a range of wavelengths for a single sample giving a spectral pattern (or profile) for each peak. The spectral absorbance for each peak was monitored over 200 - 400 nm.

Chromatogram 2 shows that the profile for 8-Cl cAMP, 8-Cl adenosine and the metabolite peak were identical. Absorbance is due to the adenine ring giving it this characteristic spectral profile. This data confirms that the metabolite peak contains an adenine ring. Furthermore, the identical retention times for the metabolite and 8-Cl adenosine (see chromatogram 1) also support that this peak is 8-Cl adenosine. It is unlikely that the identity of this metabolite peak is due to another metabolite of 8-Cl cAMP such as 8-Cl AMP or 8-Cl inosine, since these have different retention times compared to 8-Cl adenosine (Van Lookeren Campagne *et al.*, 1991).

Chromatogram 2:

UV absorbance (normalised to maximum absorbance; 100%) of 8-Cl cAMP (BLUE), 8-Cl adenosine (RED) and an 8-Cl cAMP metabolite (GREEN; see section 3.22) over a wavelength range of 200 - 400 nm.



3.23 CONCENTRATION DEPENDENT FORMATION OF METABOLITE WITH cAMP ANALOGUES

3.23.1 Introduction:

Data presented in section 3.14 demonstrated that 8-Cl cAMP was a more potent antiproliferative agent than 8-Br cAMP cultured in serum containing conditions (compare Figure 12 &13). Evidence presented in this thesis suggest that the antiproliferative effect of 8-Cl cAMP is due to production of a metabolite, therefore suggesting that the antiproliferative effect of 8-Br cAMP may also be due to a metabolite. The different potencies of these two analogues could be explained by a higher rate of metabolism for 8-Cl cAMP compared to 8-Br cAMP.

3.23.2 Rate of 8-Cl cAMP and 8-Br cAMP Metabolism In HITS Medium Containing 10% FCS (v/v):

A range of 8-Cl cAMP and 8-Br cAMP concentrations were incubated in HITS medium containing 10% FCS (v/v) for 72 hours at 37 °C. The medium was filtered (section 2.11.3) before HPLC analysis (section 2.11.4). Pure 8-Br adenosine was not available to construct a calibration plot for peak area against concentration. However, since 8-Cl adenosine and 8-Cl cAMP gave similar calibration plots (see Figure 21), and the only difference between the two cAMP analogues is the halogen, then the molar absorbance for 8-Br cAMP could be used to calculate a concentration for the metabolite peak areas.

HPLC analysis of medium incubated with 8-Br cAMP showed two peaks; one known to be 8-Br cAMP and the other assumed to be 8-Br adenosine. The concentration of both 8-Cl adenosine and

the putative 8-Br adenosine, shown in Figure 23, demonstrate that similar concentrations of both metabolites were formed in the medium. Furthermore, this production appeared to be dependent on the starting concentration of the cAMP analogue; rate of hydrolysis works out to be-

85 pM metabolite/ 390 pM cAMP analogue/ minute

(Derived from Figure 23).

These data suggest that metabolism of both analogues are equivalent and therefore does not provide an explanation for their different potencies. Nevertheless, comparison of the growth inhibition data for different concentrations of analogue (Figure 12 & 13) with this data (Figure 23) suggest that the concentration of the metabolite correlates with the level of growth inhibition of MCF-7 cells.

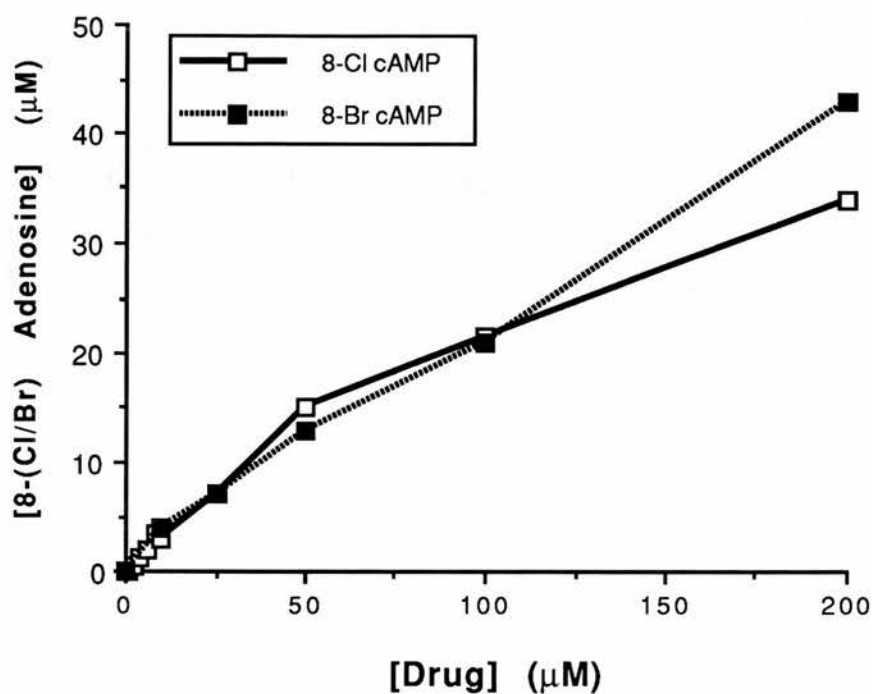


FIGURE 23:

The formation of 8-Cl adenosine and 8-Br adenosine in HITS medium containing 10% FCS (v/v) plus cAMP analogue incubated for 72 hours at 37 °C.

Medium was filtered through a 10 kDa Centricon filter before analysis by HPLC.

3.24 EFFECT OF INCUBATION TIME ON METABOLITE FORMATION

Data from section 3.18 suggested that IF-s activated 8-Cl cAMP, enabling the drug to inhibit cellular proliferation in serum-free conditions, and that the degree of inhibition was time dependent. To determine if this growth inhibition was due to formation of 8-Cl adenosine these filtrates were analyzed by HPLC (section 2.11.3 & 2.11.4) .

8-Cl adenosine was detected in these samples. The concentration increased from 3 hours to 12 hours (Figure 24). Metabolite concentration in samples incubated for longer periods did not appear to increase further, apart from the 72 hour incubation. Even though no relationship appears to exist for time and concentration of metabolite for this data, the presence of metabolite in the media still correlates with the effect on cellular proliferation (section 3.18). Furthermore, the medium incubated at 4 °C for 72 hours had 93% less detectable 8-Cl adenosine compared to the sample incubated at 37 °C.

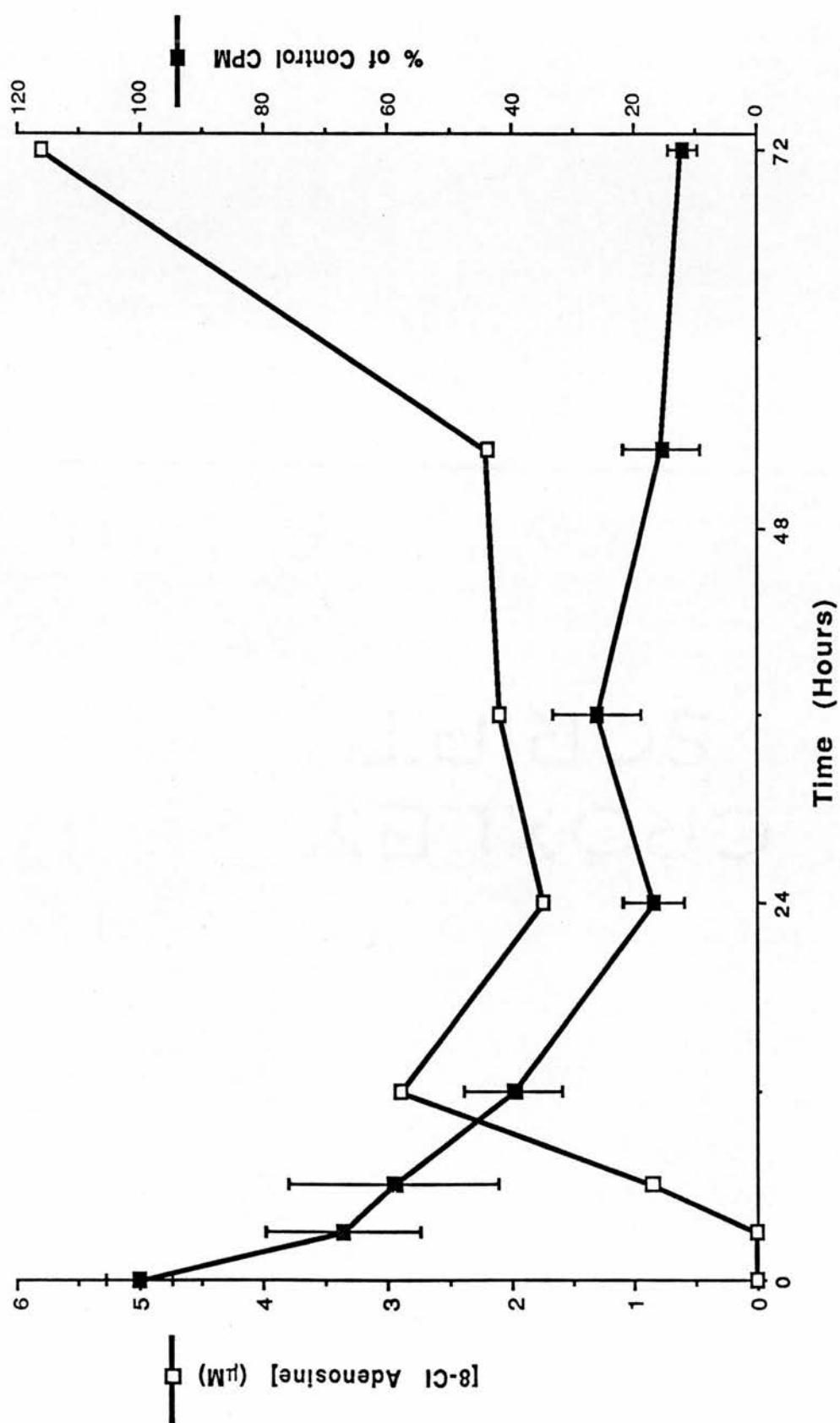


FIGURE 24:

The formation of 8-Cl adenosine in HITS medium containing 10% FCS (v/v) plus 8-Cl cAMP (10 μM) incubated at 37 °C for varying time periods. This data is also displayed with the thymidine uptake data from section 3.18 (see Figure 18 legend).

3.25 IBMX INHIBITS THE GENERATION OF 8-Cl ADENOSINE FROM 8-Cl cAMP BY SERUM

Addition of IBMX to culture medium containing serum reduced the level of inhibition by 8-Cl cAMP (section 3.19). To determine if this protective effect of IBMX was the result of reducing the concentration of 8-Cl adenosine formed, HITS medium containing 10% FCS (v/v) was incubated for 72 hours with either 100 μ M or 10 μ M 8-Cl cAMP in the presence or absence (control) of a range of IBMX concentrations. Samples were prepared (section 2.11.3) and analyzed by HPLC (section 2.11.4).

Incubation of 100 μ M 8-Cl cAMP in the presence of 0.025 mM IBMX resulted in the reduction of 8-Cl adenosine from 19 μ M to 7 μ M (Figure 25). A further, but smaller, decrease in the concentration of 8-Cl adenosine was observed with increasing concentrations of IBMX. Furthermore, concentrations above 0.025 mM resulted in no detectable 8-Cl adenosine when a starting concentration of 10 μ M 8-Cl cAMP was incubated in the medium (Figure 25).

These data suggest that the protective effect of IBMX against the antiproliferation response of 8-Cl cAMP (section 3.19; Figure 19) is due to reduction in the concentration of 8-Cl adenosine.

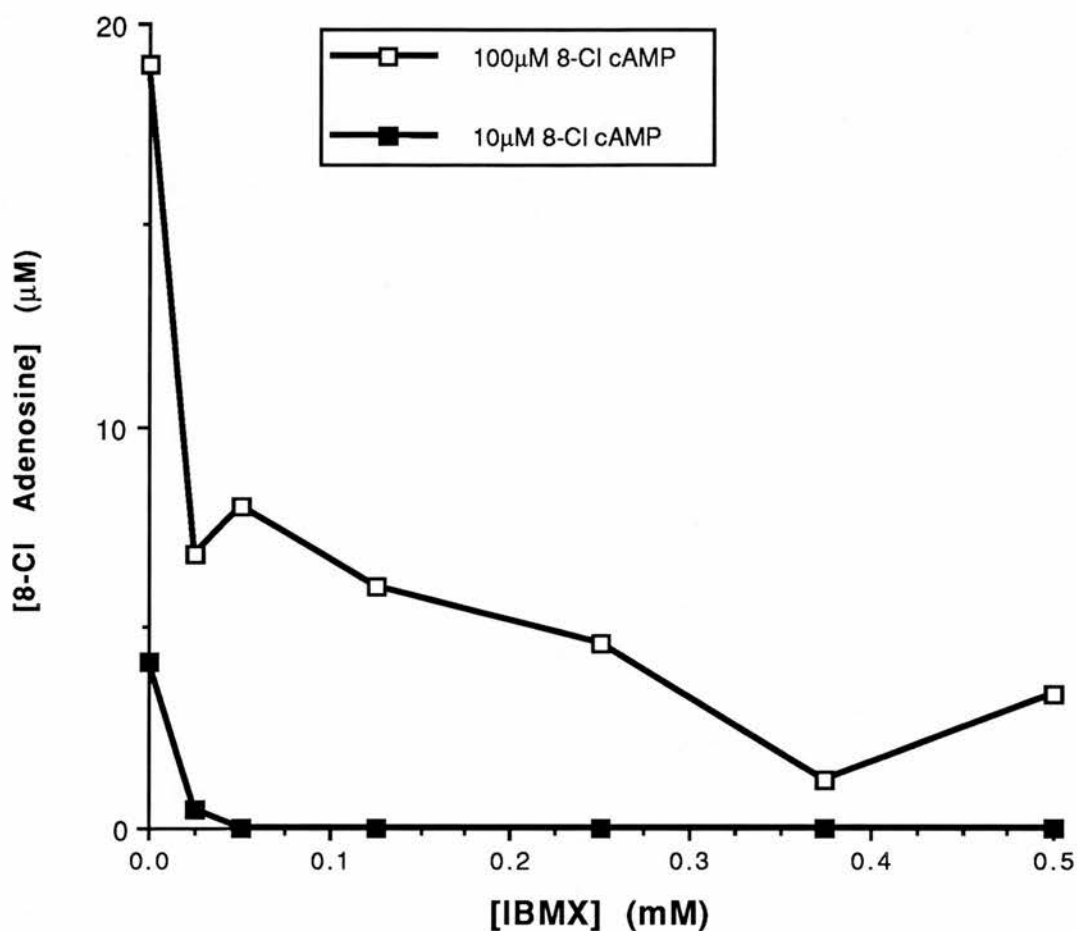


Figure 25:

The Effect of isobutylmethylxanthine (IBMX) on the formation of 8-Cl adenosine in HITS medium containing 10% FCS (v/v) and 8-Cl cAMP incubated at 37 °C for 72 hours.

Medium was filtered through a 10 kDa Centricon filter before analysis by HPLC.

3.26 THE EFFECT OF PDE ON METABOLISM OF 8-Cl cAMP IN SERUM-FREE MEDIUM

Addition of the crude PDE preparation to HITS medium induced an 8-Cl cAMP inhibition on MCF-7 cells (section 3.20). To determine if the growth inhibitory effect produced by crude PDE was due to 8-Cl AMP formation, HITS medium containing PDE (0.01 - 0.1 units / ml) and 8-Cl cAMP (100 μ M) was incubated for 72 hours at 37 °C. The media was then prepared (section 2.11.3) for HPLC analysis (section 2.11.4). Standard for 8-Cl AMP was not available. Nevertheless, the formation of a new metabolite peak such as 8-Cl AMP can be monitored because of different retention times for metabolites, but the identity of additional peaks can only be assumed.

At 0.04 units/ml of PDE all detectable 8-Cl cAMP was completely metabolised; 8-Cl adenosine was also detectable. However, higher concentrations of PDE resulted in a decrease in the concentration of 8-Cl adenosine. Formation of two new metabolite peaks were identified that had shorter retention times to the column than that of 8-Cl cAMP or 8-Cl adenosine. These two peaks were not detected in previous samples containing serum and nor were they detected in medium containing PDE alone. Based on the retention times report by Van Lookeren Campagne's group (1991) for 8-Cl cAMP metabolites, the additional peaks eluted from the column were probably 8-Cl AMP and 8-Cl inosine.

These data confirm that this crude PDE metabolizes 8-Cl cAMP to more than just 8-Cl AMP, suggesting that the preparation contains other metabolising enzymes including nucleotidase due to the formation of 8-Cl adenosine.

3.27 METABOLITE OF 8-Cl cAMP MEDIATED BY FCS

FRACTIONS

Gel filtration of concentrated FCS yielded a number of fractions that contained a high activity of IF-s (section 3.17). To establish if the activity from these fraction was proportionate to the formation of 8-Cl adenosine, fractions from these two columns were added at 10% (v/v) to medium containing HITS. 8-Cl cAMP (50 μ M) was incubated for 72 hours in this media and analyzed for metabolite formation by HPLC (section 2.11.3 & 2.114).

Figures 26 and 27 show the concentration of 8-Cl adenosine formed for each fraction, combined with the data previously shown for the protein concentration of the fractions and the percentage of thymidine incorporation (section 3.17; Figures 16 & 17, respectively). Those fractions that were demonstrated to contain the major IF-s activity also appeared to have the highest concentrations of 8-Cl adenosine.

These data show that the fractions, thought to contain the highest concentrations of IF-s, have the highest concentrations of PDE and 5'-nucleotidase activity. This suggests that the identity of IF-s could be serum borne PDE and 5'-nucleotidase.

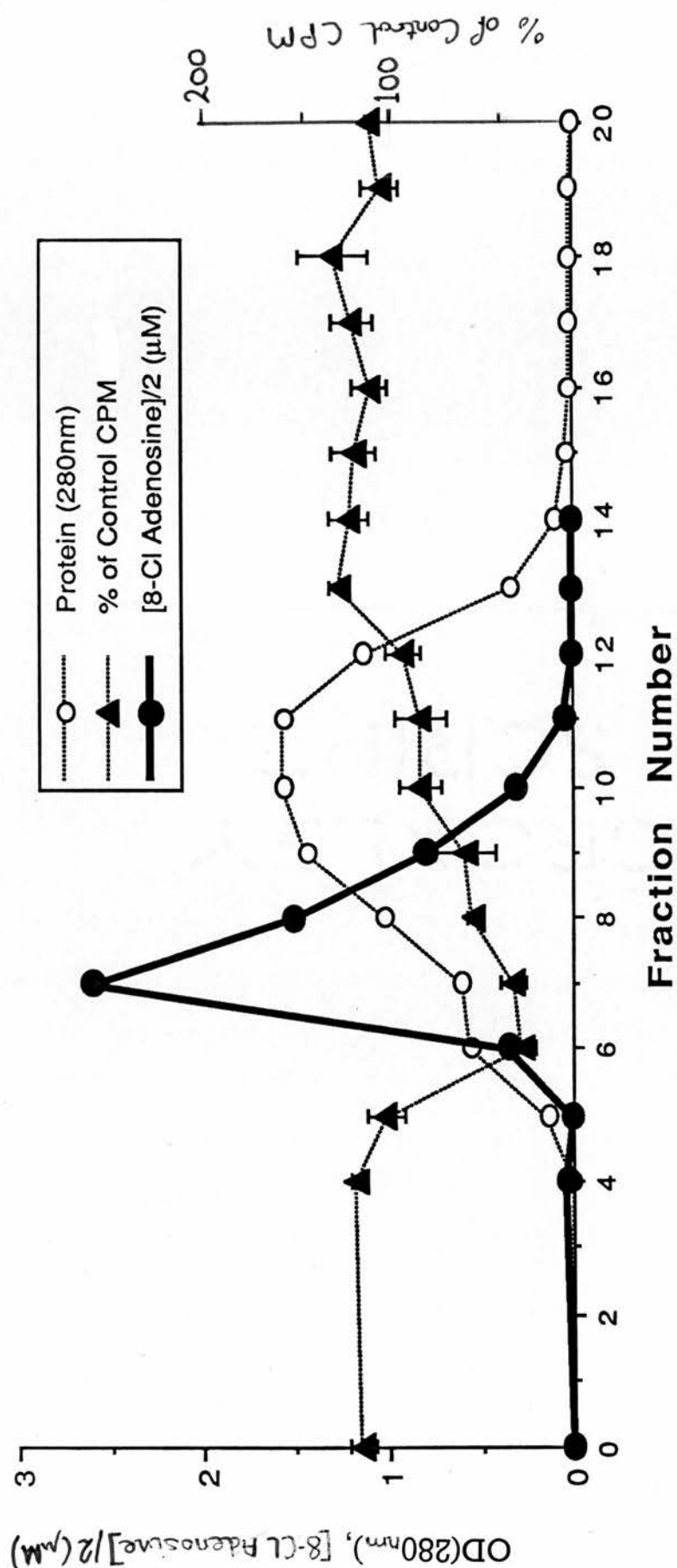


FIGURE 26:

The formation of 8-Cl adenosine in HITS medium containing 10% FCS fractions (v/v) (section 3.17.2) plus 8-Cl cAMP (50 μ M) incubated at 37 $^{\circ}$ C for 72 hours.

Medium was filtered through a 10 kDa Centricon filter before analysis by HPLC.

Thymidine incorporation and protein concentration data from figure 16 (see legend).

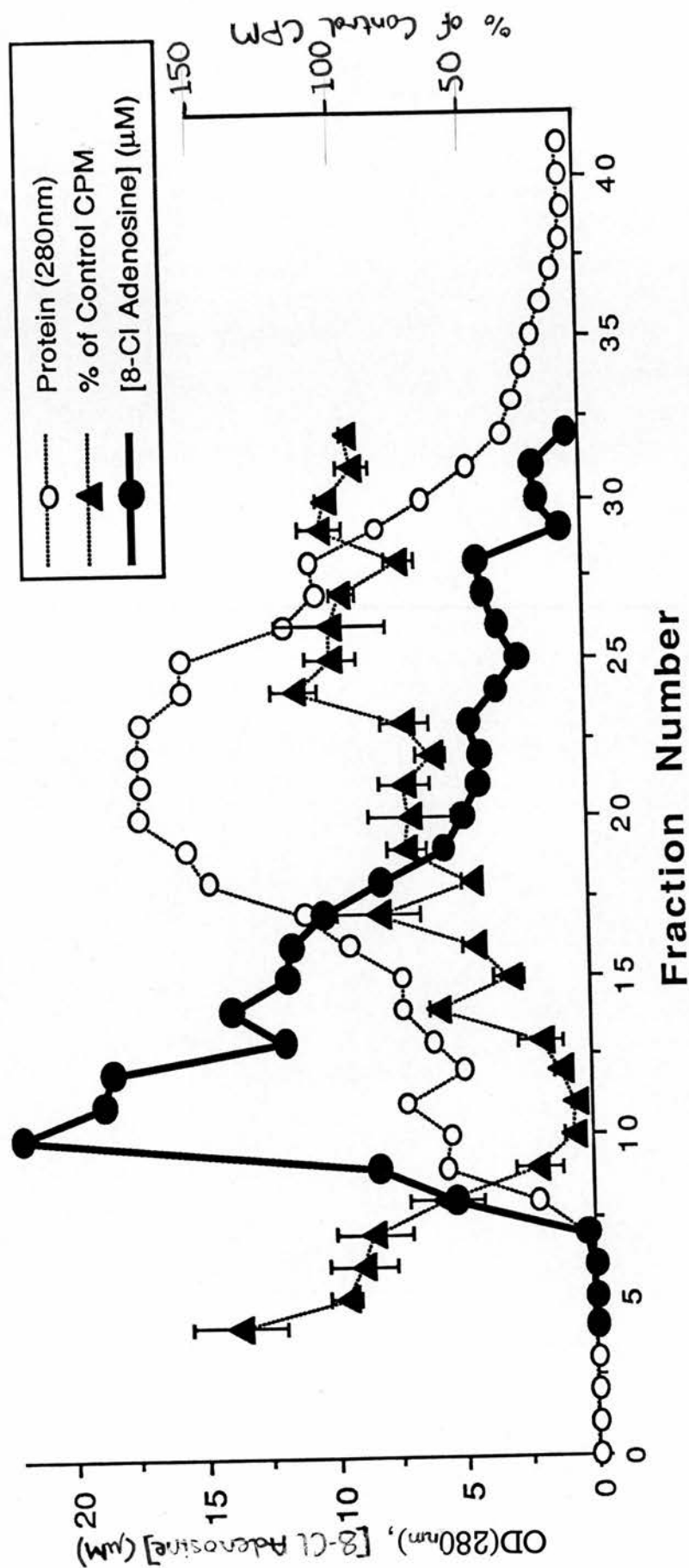


FIGURE 27:

The formation of 8-Cl adenosine in HITS medium containing 10% FCS fractions (v/v) (section 3.17.3) plus 8-Cl cAMP (50 μM) incubated at 37 °C for 72 hours. Medium was filtered through a 10 kDa Centricon filter before analysis by HPLC.

Thymidine incorporation and protein concentration data from figure 17 (see legend).

3.28 METABOLISM OF 8-Cl cAMP BY OTHER SERA

3.28.1 Human Serum:

Fresh human blood was used to prepare serum to determine if it possessed IF-s activity and metabolized 8-Cl cAMP. The human serum was added to HITS medium to give different concentrations and incubated with 8-Cl cAMP (100 μ M) at 37 °C for 72 hours. The media was then prepared (section 2.11.3) and analyzed by HPLC (section 2.11.4). In addition, the media was also used for the thymidine uptake assay (section 2.1.6) in the presence or absence (control) of 8-Cl cAMP (10 μ M).

Figure 28 shows the combined data for thymidine uptake and concentration of 8-Cl adenosine. The degree of inhibition of thymidine incorporation appears to be dependent on the concentration of human serum in the medium. Furthermore, this growth inhibition correlated with the concentration of 8-Cl adenosine. A maximum of approximately 60% inhibition was demonstrated with 10%; this compares with approximately 80% for FCS and may be explained on the bases of 8-Cl adenosine formation (see 3.28.2).

3.28.2 Mouse Serum:

Using nude mouse serum, a 10% (v/v) solution in HITS medium was incubated at 37 °C with 8-Cl cAMP (100 μ M) for 72 hours.

By HPLC analysis 31 μ M of 8-Cl adenosine was detected. This compares with a value for 10% (v/v) FCS and human serum of 22 μ M \pm 3.5 (n=3) and 10 μ M, respectively.

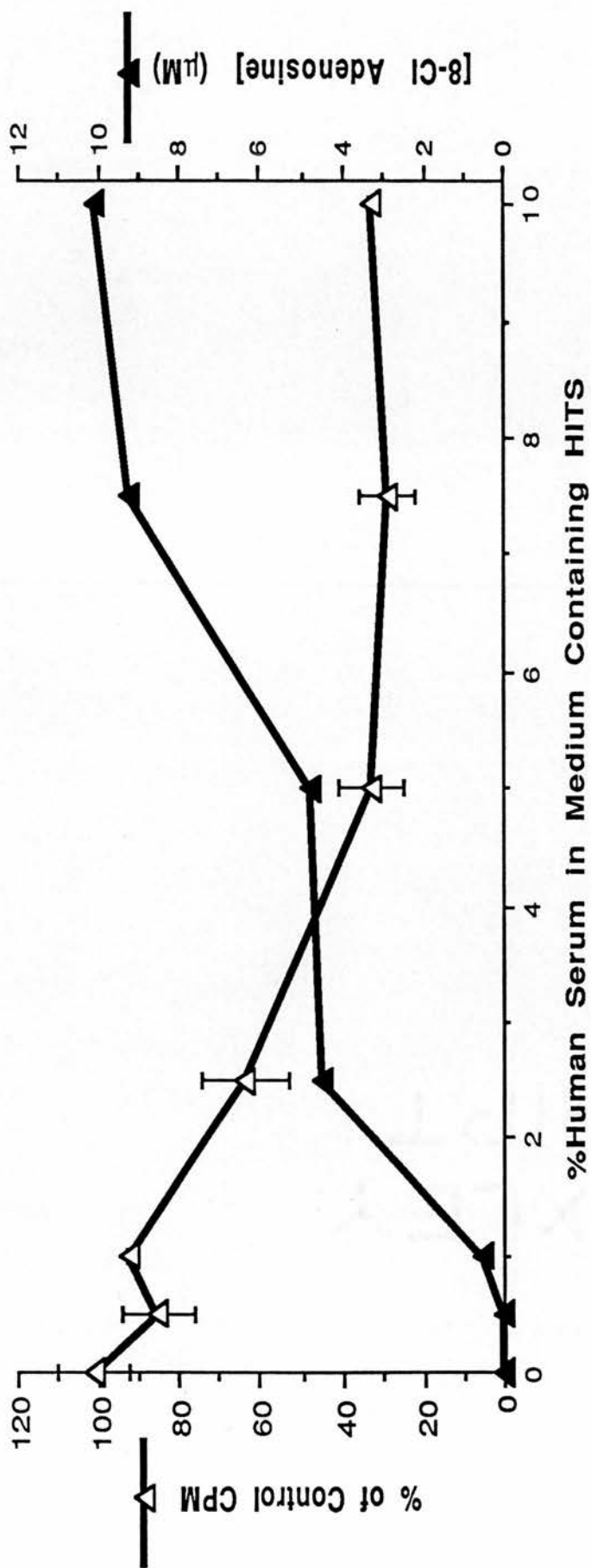


FIGURE 28:

The effect of 8-Cl cAMP (10 μ M) on the incorporation of [methyl- 3 H] thymidine by MCF-7 cells cultured in HITS medium containing different concentrations of human serum (v/v) for 78 hours. Control cells were not treated with 8-Cl cAMP (i.e. 100%). Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

The formation of 8-Cl adenosine in HITS medium containing different concentrations of human serum (v/v) and 8-Cl cAMP (100 μ M), incubated at 37 $^{\circ}$ C for 72 hours. Medium was filtered through a 10 kDa Centricon filter before analysis by HPLC.

3.29 INHIBITION OF THYMIDINE UPTAKE BY **8-Cl ADENOSINE**

To demonstrate that 8-Cl adenosine was toxic to MCF-7 cells an experiment was set up adding concentrations of 8-Cl adenosine or 8-Cl cAMP to HITS medium or HITS medium containing 10% FCS (v/v) and assayed with MCF-7 cells for inhibition of thymidine uptake (section 2.1.6).

8-Cl adenosine dramatically inhibited thymidine uptake both in HITS and serum containing medium (Figure 29). 8-Cl cAMP, as previously demonstrated, did not inhibit uptake in HITS medium but inhibition of thymidine incorporation increased with respect to concentration of drug added to serum containing medium.

These data demonstrate that 8-Cl adenosine inhibits proliferation of MCF-7 cells in HITS medium, in the presence or absence of serum. The apparent IC_{50} for 8-Cl adenosine in HITS medium containing serum is equivalent to the value quoted by Katsaros *et al.* (1988) of 1 μ M (see section 3.2.1). Furthermore, the inhibition of MCF-7 cells cultured in HITS medium containing 10% FCS (v/v) by 8-Cl adenosine was more effective at equimolar concentrations of 8-Cl cAMP.

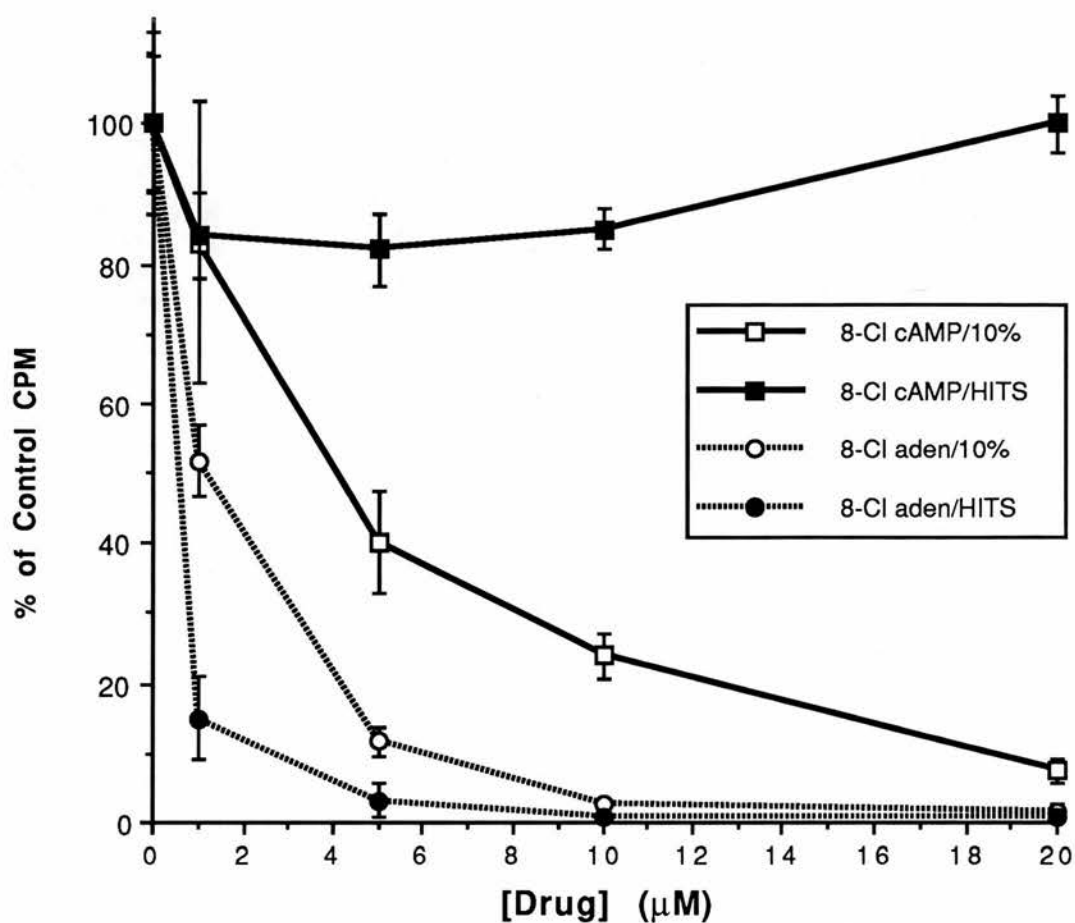


FIGURE 29:

The effect of different concentrations of 8-Cl adenosine or 8-Cl cAMP on the incorporation of [methyl-³H] thymidine with MCF-7 cells cultured for 78 hours in HITS medium either in the presence or absence of 10% FCS (v/v).

Control cells were cultured in the absence of drug (i.e. 100% incorporation).

Data are expressed as a percentage \pm standard deviation of quadruplicates, relative to incorporation by control.

SECTION 4:

DISCUSSION

4.1 Hormone-Responsive MCF-7 cells

MCF-7 cells have been used extensively as a model for hormone-responsive breast cancer since they were established in the early 1970s (Soule *et al.*, 1973). These cells can be cultured in a variety of different media and sera and many researchers have adopted different conditions for maintaining this cell line. Consequently, numerous clones of MCF-7 cells have developed which have varying population doubling times, oestrogen receptor content and responsiveness to oestrogen and tamoxifen (Butler *et al.*, 1981; Page *et al.*, 1983; Katzenellenbogen *et al.*, 1987). The initial aim of the thesis was to investigate the mechanism of antagonism by 8-Cl cAMP of oestradiol growth stimulation in MCF-7 cells, as described by Katsaros *et al.* (1988). Their MCF-7 cells were not used and it was therefore necessary to develop culture conditions and demonstrate the hormone-responsiveness of the cells used in this study. The hormone-responsiveness of these cells was demonstrated in medium containing 1% charcoal-stripped FCS (v/v) by 10^{-9} and 10^{-8} M oestradiol (section 3.1); population doubling time decreased from 4 days to 2 days. This two fold stimulation was similar compared to Katsaros's MCF-7 cells, even though their cells had a basal growth level of 8 days in medium containing 6.5% charcoal-stripped FCS (v/v). Addition of tamoxifen (5×10^{-7} - 10^{-6} M) and retinoic acid (10^{-6} M) appeared to antagonise this oestradiol stimulation of our cells. The serum concentration used by Katsaros *et al.* (1988) was not duplicated, since our MCF-7 cells showed typical responses to oestradiol, tamoxifen and retinoic acid in 1% conditions. Furthermore, this lower concentration of serum used in our culture system ensured that endogenous steroids (not removed by

charcoal-stripping) and other growth promoting factors were kept to a minimum, therefore allowing closer examination of the effects that exogenous agents had on the proliferation of the cells in culture.

4.2 Mechanism Of Tamoxifen Antagonism

Tamoxifen was originally developed as a competitive inhibitor for the binding of oestradiol to its receptor, thus preventing activation and eventual stimulation of proliferation. However, there is accumulating evidence suggesting that tamoxifen can modulate proliferation other than by antagonism of the oestradiol response. Butler *et al.* (1981) demonstrated that in the absence of added oestradiol, growth inhibition of MCF-7 cells by tamoxifen (1 μ M) was more effective at low concentrations of charcoal-stripped serum (0.1 - 1% (v/v)) than at higher concentrations of serum (10% (v/v)). They also demonstrated that the degree of stimulation by oestradiol (5×10^{-8} M) diminished as the concentration of serum increased. Vignon *et al.* (1987) also demonstrated that hydroxytamoxifen (1 μ M) inhibited the proliferation of MCF-7 cells in the complete absence of oestradiol in the medium. However, this antiproliferative effect did require the presence of oestradiol receptors, since this effect could not be demonstrated in cells lacking oestradiol receptor. Other reports have also suggested that addition of tamoxifen can stimulate MCF-7 cells and alter expression of gene products that do not appear to be regulated by oestradiol (May *et al.*, 1989; Westley *et al.*, 1989). Our MCF-7 cells had tamoxifen regulated proteins that did not appear to be controlled by oestradiol (section 3.6.3). Treatment of MCF-7 cells with tamoxifen (1 μ M)

resulted in the expression of three proteins detected by Western blotting with the monoclonal antibody BA17 against cytokeratin 19. Only two of these proteins appeared to be expressed in the presence or absence of oestradiol and expression was much lower than for tamoxifen treated MCF-7 cells. Similar patterns of protein staining in control cells and oestradiol treated cells suggest that oestradiol does not affect the expression of these proteins and tamoxifen can. The mechanism of this is unknown; however, expression of these three proteins could be a consequence of the antiproliferative effect of tamoxifen. On the other hand, the antiproliferative effect of retinoic acid does not induce the same changes in expression of these proteins as does tamoxifen.

4.3 8-Cl cAMP Antagonism of Oestradiol Stimulation in MCF-7 cells

Katsaros *et al.* (1988) demonstrated that 8-Cl cAMP (1 μ M) produced a 50% growth inhibition of MCF-7 cells stimulated by oestradiol (10^{-8} M) after a period of 5 days. In contrast, addition of ten times the concentration of 8-Cl cAMP (10 μ M) to our MCF-7 cells in 1% FCS conditions produced no dramatic alteration in proliferation in the presence or absence of oestradiol (section 3.2.2). Hypothetically, according to observations by Butler *et al.* (1981) with tamoxifen, a lower concentration of serum and a higher concentration of 8-Cl cAMP would have been expected to have a greater inhibition of MCF-7 cells after 5 days than that reported by Katsaros *et al.* (1988). Using HITS as a serum substitute in the medium, MCF-7 cells had a doubling time of 2 days in culture and did not appear to respond to exogenous

oestradiol or 8-Cl cAMP (section 3.11). This apparent loss of hormone-responsiveness in serum-free conditions has previously been observed by Katzenellenbogen *et al.* (1987). They cultured MCF-7 cells for 5 to 6 months in phenol red free and charcoal-stripped FCS conditions, essentially devoid of oestradiol. Addition of oestradiol to the cells after this period of time did not produce a hormone response in cells that were initially responsive. However, an antiproliferative effect by 8-Cl cAMP was demonstrated with MCF-7 cells used in this study when cultured in conditions containing 10% FCS (v/v), charcoal-stripped or unstripped (section 3.3). Furthermore, as the concentration of serum in the medium decreased then so did the level of inhibition by 8-Cl cAMP (10 μ M) (sections 3.3, 3.11 & 3.13). In addition, the level of growth inhibition by 8-Cl cAMP appeared to be similar in the presence or absence of oestradiol, regardless of the serum concentration in the medium (section 3.3). These data suggested a serum-dependent 8-Cl cAMP growth inhibition of MCF-7 cells, but no direct antagonism of the oestradiol growth stimulation. Compared to the report of Katsaros *et al.* (1988); they did not investigate the effects of 8-Cl cAMP on MCF-7 cells cultured in the absence of oestradiol or in serum-free conditions. It is distinctly possible that the observed inhibition of growth was misconstrued as antagonism, and they may have demonstrated similar data as presented in this thesis if growth inhibition by 8-Cl cAMP was studied in the absence of oestradiol or serum-free conditions.

4.4 p21, Kinase A Regulatory Subunits and Cell Cycle Analysis

A decrease in the expression of p21 has been observed with tumours and cell lines growth inhibited by cAMP analogues. This concomitant decrease in p21 on growth inhibition was also associated with a change in the expression ratio of RI and RII subunits of protein kinase A (see Introduction). However, in experiments presented here, alteration in the expression of p21 and RI : RII levels in MCF-7 cells growth inhibited by 8-Cl cAMP were not detected (section 3.7 & 3.8), in contrast to previous reports by Tagliaferri *et al.* (1988) and Katsaros *et al.* (1988). It is possible that this clone of MCF-7 cells is sufficiently different from these authors cells to alter the mechanism by which 8-Cl cAMP modulates p21 and RI : RII expression ratio. This would suggest that the change in expression of p21 and RI : RII is not a prerequisite for growth inhibition by 8-Cl cAMP. The cell cycle progression data (section 3.9) also suggest differences in the mechanism. Growth inhibition of MCF-7 cells by 8-Cl cAMP produced a block in G2 + M phase which has not been previously observed (Tagliaferri *et al.*, 1988).

Despite the differences, the antiproliferative effect of 8-Cl cAMP was qualitatively similar in our cells to previous reports. However, this effect appeared to be dependent on the concentration of serum in the culture medium, an observation not reported. This serum-dependent 8-Cl cAMP growth inhibition was not a phenomenon exclusive to MCF-7 cells since it was also demonstrated in other cell lines (section 3.12).

4.5 Serum-Dependent 8-Cl cAMP Growth Inhibition

The observation that 8-Cl cAMP (10 μ M) growth inhibition was dependent on the concentration of serum in medium led to the thesis that a serum-borne component was required in combination with 8-Cl cAMP to inhibit cellular proliferation. This putative serum factor(s) (IF-s) was also present in charcoal-stripped serum (section 3.3). Many studies (including Katsaros *et al.*, 1987; Tagliaferri *et al.* 1988; Tortora *et al.*, 1988; Guadagni *et al.*, 1991) using the site-selective analogue 8-Cl cAMP on cancer cells have assayed for growth inhibition in medium containing different serum concentrations from 5 to 10% FCS (v/v). The growth inhibition reported by these authors was considered entirely due to the affect of the drug on the cell, however, a serum-dependent 8-Cl cAMP growth inhibition may have been present. There are reported cases of site-selective analogues affecting the Harvey murine sarcoma virus transformed NIH/3T3 fibroblast line in serum-free medium (Tagliaferri *et al.*, 1985; Tagliaferri *et al.*, 1988). It is therefore likely that there are some cancer cell lines, like these fibroblasts, that respond directly to cAMP analogues without the need for the putative serum factor(s) IF-s.

4.6 Characterisation of IF-s

The data in section 3.15 demonstrated that IF-s in FCS was heat labile, suggesting that IF-s might be a protein. Heat inactivation appeared to occur between the temperatures of 56 °C and 60 °C. We attempted initially to isolate a serum fraction enriched in IF-s. Early experiments demonstrated that IF-s could

not pass through the 100 kDa Centricon filter (section 3.16) and suggested that its molecular weight was greater than 100 kDa. However, PAGE analysis of the concentrate and filtrate suggested that these filters have poor fidelity under these conditions in selectively isolating proteins of molecular weights according to the filters nominal cut off size. Only a small proportion of the serum albumin (molecular weight 67 kDa) passed through the 100 kDa cut off filter. It is possible that because of the high concentration of albumin in the serum, the filter could have become blocked. Alternatively, albumin may be associated with other proteins, including itself, giving an apparent molecular weight in serum of greater than 100 kDa during filtration. Because, the reliability of these molecular weight cut off filters was in doubt, it was in any case not possible to conclude that the molecular weight of IF-s is greater than 100 kDa.

Gel filtration chromatography of the 10 kDa concentrate, through two individual columns, gave profiles with the majority of the inhibitory activity appearing in the early fractions (section 3.17). IF-s was not eluted in a sharp peak; activity slowly diminished in fractions eluted after the major activity. This may simply reflect the low resolving power of the column used. Alternatively, IF-s may be composed of species of heterogeneous molecular weights. The largest protein peak eluted from the column was probably due to the most abundant protein in the serum, albumin and fractions eluted from the column before this peak have a higher molecular weight. The majority of IF-s was eluted in these early fractions, well before albumin, suggesting that it has a molecular weight well in excess of 67 kDa.

The experiment in section 3.18 demonstrate that preincubating 8-Cl cAMP in serum containing medium produced a time-

dependent activation of the drug. This activated species could then be separated from IF-s by 10 kDa ultrafiltration. The facts that: IF-s appeared to activate 8-Cl cAMP in a time-dependent manner; the rate of activation was lower at 4 °C; IFs is heat labile (section 3.15), strongly implies that IF-s might be an enzyme(s).

NB: It was at this stage of the thesis that the data of Van Lookeren Campagne *et al.* (1991) were published.

4.7 Metabolism of 8-Cl cAMP

In a recent article (Van Lookeren Campagne *et al.*, 1991), evidence was presented that 8-Cl cAMP (100 µM) was metabolised in medium containing 10% FCS (v/v) incubated for 3 days at 37 °C, in the absence of cells. The authors suggested that the endogenous phosphodiesterases and 5'-nucleotidases in serum were converting the drug to the toxic metabolite 8-Cl adenosine. In contrast, Tagliaferri *et al.* (1988) reported that no detectable 8-Cl adenosine, as measured by HPLC, was present in 10% FCS (v/v) medium used to culture HT-29 cells treated with 8-Cl cAMP (50 µM) for 48 hours. Van Lookeren Campagne's group stated that this discrepancy could have arisen from the use of inappropriate methodology for HPLC analysis of 8-Cl adenosine. They stated that the pH buffer (pH 3.8) used by Tagliaferri *et al.* (1988) resulted in a net positive charge on 8-Cl adenosine, which therefore would not be retained by the strong ion exchange column. Van Lookeren Campagne's group concluded that 8-Cl cAMP growth inhibition of chinese hamster ovary (CHO) cells and Molt-4

cells cultured in 10% FCS (v/v) medium was mediated through 8-Cl adenosine. Again in contrast, however, Tortora *et al.* (1988) reported that addition of phosphodiesterase inhibitors, IBMX (0.5 mM) or theophylline (0.1 mM), did not affect the inhibitory effect of 8-Cl cAMP (5 - 20 μ M) on a spectrum of leukaemic cells including Molt-4 cells, cultured in 10% FCS (v/v) media. The fact that the inhibitory activity of 8-Cl cAMP was not affected in these conditions suggested to the authors that phosphodiesterases were not metabolizing the drug. On the other hand, if 8-Cl adenosine was responsible for the inhibitory response of 8-Cl cAMP then addition of phosphodiesterase inhibitors should have prevented the growth inhibitory effect of 8-Cl cAMP in these culture conditions. However, addition of IBMX (0.025 - 0.5 mM) to our MCF-7 cells cultured in 10% FCS (v/v) did not enhance the 8-Cl cAMP response but reduced it (section 3.19). Furthermore, cells cultured in HITS were growth inhibited by 8-Cl cAMP in the presence of phosphodiesterase (section 3.20). These data appear to contradict their observation. Using Van Lookeren Campagne's HPLC method, 8-Cl adenosine was detected in serum containing medium with only 10 μ M 8-Cl cAMP (section 3.21.3). This demonstrated that a concentration of 10 μ M 8-Cl cAMP, previously suggested by Tagliaferri *et al.* (1988) and Cho-Chung (1989) to be too low for metabolism by phosphodiesterases, was metabolised to 8-Cl adenosine. The concentration of 8-Cl adenosine in the medium also appeared to correlate with degree of growth inhibition of MCF-7 cells induced by 8-Cl cAMP (section 3.21.3; further discussed in section 4.9).

4.8 Adenosine Toxicity

Adenosine is a purine nucleoside that is an intermediate in the pathway of purine nucleotide degradation. In addition to adenosine's physiological role in cellular biochemistry (eg. AMP, ATP), high concentrations in the range of 1 - 1000 μM are known to be toxic to mammalian cells (Green & Chan, 1973; Ullman *et al.*, 1976; Hershfield *et al.*, 1977). The precise mechanism of adenosine toxicity is not clearly understood but it has been shown that high concentration can affect the activity of enzymatic reactions involved in the synthesis and degradation of purines and pyrimidines. Studies have shown that adenosine can reduce the activity of orotate phosphoribosyltransferase and this leads to depletion of the pyrimidine nucleotide pool (Planet & Fox, 1977; Ishii & Green, 1973; Kaukel *et al.*, 1972). Adenosine has also been reported to reduce the intercellular concentration of 5-phosphoribosyl-1-pyrophosphate (PRPP) (Snyder & Seegmiller, 1976; Planet & Fox, 1976). PRPP is an essential substrate in purine and pyrimidine *de novo* biosynthesis, the purine salvage pathways and pyrimidine nucleotide synthesis (Fox & Kelly, 1971). A decrease in PRPP can, therefore, have a profound metabolic effect on the cell, especially DNA synthesis.

The toxicity of 8-Cl adenosine was demonstrated with a range of concentrations (1 - 20 μM) with MCF-7 cells cultured in the presence or absence of serum (section 3.29). This compares with reports that demonstrated toxicity with 2 and 5 μM (Katsaros *et al.*, 1988; Tagliaferri *et al.*, 1988). These authors also showed that 8-Cl adenosine induce a cell cycle progression block in S phase of LS-174T cells, and this growth inhibition did not result in a change in p21 and R1 : RII levels. These effects of 8-Cl

adenosine appear to be similar to those described for 8-Cl cAMP in this thesis. Though the limited availability of 8-Cl adenosine prevented studies on p21, R1, R11 and cell cycle analysis, the data presented here do suggest that the 8-Cl cAMP response is mediated by the metabolite 8-Cl adenosine, as suggested by Van Lookeren Campagne's group. They also showed that 8-Cl adenosine inhibited their CHO cells, but an equimolar concentration of 8-Cl cAMP proved more potent in serum containing medium. The authors attempted to explain this as due to 8-Cl cAMP acting on a reservoir for the release of cellular adenosine. Our MCF-7 cells, on the other hand, appear to be two fold more sensitive to 8-Cl adenosine than 8-Cl cAMP (section 3.29). If adenosine was released from intracellular reservoirs as a result of added 8-Cl cAMP, it did not appear to play a role in the inhibition of our cells since no inhibition was observed with cells cultured in HITS medium with drug. In serum containing culture conditions, however, addition of a concentration of 8-Cl adenosine is probably more effective than 8-Cl cAMP at inhibiting the MCF-7 cells because production of the metabolite appears to be time-dependent (sections 3.18 & 3.24).

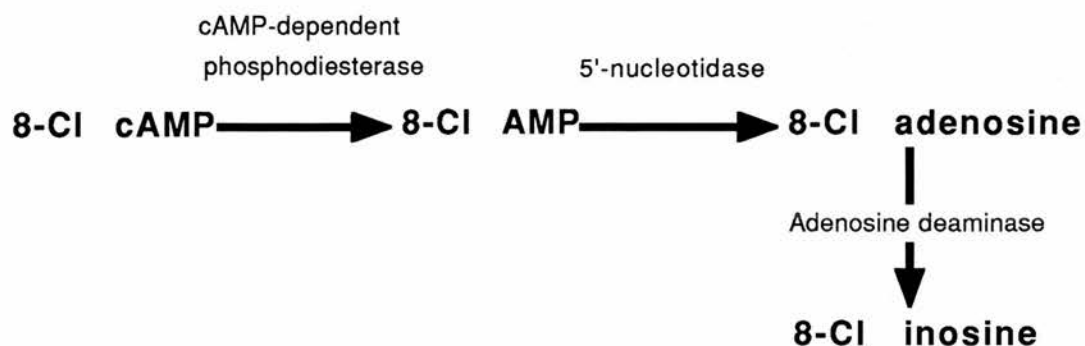
The conflicting data from Van Lookeren Campagne *et al.* (1991) and this thesis, compared to other workers on the action of 8-Cl cAMP is difficult to explain. The two serum-free studies which demonstrated a cAMP analogue^{response} cannot be explained by metabolism of the drug in the media (Tagliaferri *et al.*, 1985; Tagliaferri *et al.*, 1988). However, it is possible that the analogues were hydrolysed by cellular phosphodiesterases. All other studies have been performed in media containing serum. Van Lookeren Campagne's group (1991) studied different batches of FCS and other sera for hydrolysis of 8-Cl cAMP and found that they all

degraded the drug, but the rate of hydrolysis varied: 2% hydrolysis for human serum; 28 - 72% hydrolysis for other sera. The authors also showed that heat-inactivation of FCS reduced the level of activity by at least 6 fold. Though only heat-inactivated FCS was used in this thesis, the data presented in section 3.15 support this observation, since the activity of the hydrolysing enzymes in serum was denatured at 60 °C. It is possible that other workers used sera with a low hydrolysis activity or denatured all hydrolysing activity by heat-inactivating their sera, therefore producing culture conditions that did not hydrolyse 8-Cl cAMP. This seems unlikely since data from this thesis show that 8-Cl cAMP in medium with heat denatured serum or HITS does not inhibit cellular proliferation (section 3.15). Alternatively, it is possible that other studies used cells that were sensitive to 8-Cl cAMP. This also seems improbable since a total of five established cell lines, including three lines previously demonstrated to be inhibited by 8-Cl cAMP, appeared to be insensitive to the drug in the serum-free conditions (section 3.12). Previous reports of growth inhibition by 8-Cl cAMP was probably due to formation of 8-Cl adenosine. However, the antiproliferative effect may have been due to not only 8-Cl adenosine but also 8-Cl cAMP if the cell line was directly sensitive to the analogue. The reported changes in p21 and R1 : R11 expression ratio by 8-Cl cAMP, which could not be demonstrated in our MCF-7 cells, may have been due to a direct affect of 8-Cl cAMP on cells.

4.9 Phosphodiesterases (PDE) And 5'-Nucleotidases

Early literature concerning PDE degradation of cAMP analogues typically refers to the PDE located within the cell and not to PDE in mammalian sera, which have been recognised since the early 1970s (Asano & Hidaka, 1975).

Cells contain three basic types of cAMP-dependent phosphodiesterase with different affinities for cAMP. These enzymes occur as integral parts of the cell membrane and also as soluble enzymes in the cytoplasm. The soluble form is activated by Ca^{2+} acting through calmodulin (Martin, 1987; pp. 133-134). However, calmodulin is not required to activate species found in mammalian sera (Asano & Hidaka, 1975). These PDE, isolated from rabbit, rat, dog and human sera, have a molecular weight range from 150 to 340 kDa. Asano and Hidaka (1975) studied the enzyme kinetics of these and calculated the K_m values for cAMP hydrolysis which ranged from 0.81 - 76 μM . Furthermore, they calculated the PDE activity with 0.4 μM cAMP, which ranged from 1.60 - 172 pmoles/min/ml. The molecular weights for these PDE support the chromatography data (section 3.17) which suggest IF-s has a molecular weight greater than 67 kDa. Furthermore, the K_m values suggest that mammalian sera phosphodiesterase could be activated by concentrations of 8-Cl cAMP used in the literature and this thesis .



The above diagram represents the biochemical pathway for the formation of 8-Cl inosine from 8-Cl cAMP, with the appropriate enzyme for each reaction indicated. The presence of 5'-nucleotidase in serum was deduced since the only peak detected by HPLC when 8-Cl cAMP was incubated in medium containing FCS was 8-Cl adenosine (chromatogram 1; section 3.21). 5'-nucleotidases have an approximate molecular weight of 140 kDa for the native enzyme (Chuang *et al.*, 1984). The gel filtration profile is thus consistent with IF-s being comprised of serum PDE and 5'-nucleotidase.

Addition of the PDE to HITS medium with 8-Cl cAMP conferred growth inhibition on MCF-7 cells (section 3.20), and a number of metabolite peaks were detected by HPLC of the medium (section 3.26). These data indicate that the crude heart PDE preparation probably contained other metabolising enzymes, since the 8-Cl adenosine metabolite disappeared, and other metabolite peaks increased in size after incubation with 8-Cl cAMP. In contrast, the fact that no other metabolite peak, other than 8-Cl adenosine, could be detected after incubation in medium containing FCS suggest that only phosphodiesterase and 5'-nucleotidase are present, and adenosine deaminase is not. The HPLC data also

suggest that the rate-limiting step in the conversion of 8-Cl cAMP to 8-Cl adenosine is PDE and not nucleotidase, since no 8-Cl AMP could be detected. The concentration of 8-Cl adenosine detected by HPLC appeared to relate to the growth inhibitory effects demonstrated on by thymidine incorporation with MCF-7 cells. In other words, the level of growth inhibition correlated with the concentration of 8-Cl adenosine in the medium (for examples see sections 3.28.1; 3.11 & 3.21.3; 3.18 & 3.24). In addition, the apparent differences in the growth inhibitory effect of 8-Cl cAMP (10 μ M) with different sera could be explained by the relative levels of hydrolysis of 8-Cl cAMP. Human serum appeared to have a lower level of 8-Cl cAMP hydrolysis than FCS and mouse serum (section 3.28).

4.10 Toxicity of 8-Br Adenosine

Data described in section 3.23 show that 8-Br cAMP was metabolised to the same extent as 8-Cl cAMP, but 8-Br cAMP is approximately ten times less potent at growth inhibition (section 3.14).

The bromine atom is larger than the chlorine with the result that the molecule is sterically hindered to greater degree, and cannot rotate around the N9-C₁ bond between the purine and the ribose moieties (see diagram 2). The result of this hindrance for bromine is that the *syn* conformation predominates (Stolarski *et al.*, 1980). It is possible that the inhibitory properties of these cAMP analogues require them to be in the *anti* conformation, therefore making 8-Cl adenosine more potent than 8-Br adenosine. This idea of conformation implies that use of larger

halogens than bromine would decrease the toxicity of the adenosine analogue and a fluorine atom may improve toxicity because of its smaller size compared to chlorine.

4.11 Serum-Free Culture Conditions

This thesis has indicated the importance of culturing conditions for assessing the activity of drugs, especially drugs that are developed with the intention of directly affecting a biological system. It is, therefore, important to ensure that any biological effect observed as the result of drug is not due to a metabolite by the culture conditions. Many cell lines are being developed that can proliferate in serum-free conditions and these antiproliferative drugs should still have activity in these systems. In the past, serum was added to culturing conditions because it contained the essential ingredients required to maintain cellular proliferation. The components of serum essential for maintaining cultures are slowly being identified; the process is slow since different cell lines require different components and at different concentrations. Using serum-free conditions the culturing environment becomes less complicated providing a simpler system for the study of drug mechanism.

4.12 The Future of Site-Selective cAMP Analogues

A mass of data has been accumulated by Y.S. Cho-Chung's group and colleagues that suggest that 8-Cl cAMP affects the proliferation of cancer cells, with little effect on "normal" cell lines, and does so via a non-toxic mechanism (Reviewed by Cho-Chung, 1990). Detailed studies of the mechanism of action has

implicated the $R11\beta$ receptor, *ras* and *myc* oncogenes, and growth factor production. The majority of these studies were carried out in serum containing conditions. All these data appeared promising for the clinical situation as a new treatment for cancer. However, a recent preclinical pharmacology study with 8-Cl cAMP in dogs placed some doubt on the use of this analogue due to the severity of drug-induced toxicity (Tomaszewski *et al.*, 1991). Two metabolites were detected in the plasma of several dogs, 8-chloroxanthine and 8-chloro-2-hydroxyadenosine. The metabolism of 8-Cl cAMP in dogs parallel the data of Van Lookeren Campagne's group and this thesis. The specific cancer-targeting effect of these site-selective cAMP analogues appear to be lost when they are metabolised to toxic metabolites. In light of this data, the cancer lines previously reported to be sensitive to site-selective cAMP analogues should be re-assessed using serum-free medium to determine those lines, and potentially the type of cancers, that are directly responsive to the analogue as opposed to the toxic metabolite. *In-vivo*, it is more than likely that 8-Cl cAMP will be hydrolysed by patients' plasma resulting in production of the toxic metabolite. This could result in the loss of the expected cancer specificity by 8-Cl cAMP, and make it no better than other anti-cancer agents that function by cytotoxicity. Alternatively, if cancer cell proliferation is more susceptible to inhibition than normal cells, as suggested by the majority of the literature, then development of other analogues that are more resistant to phosphodiesterase may prove to be more advantageous.

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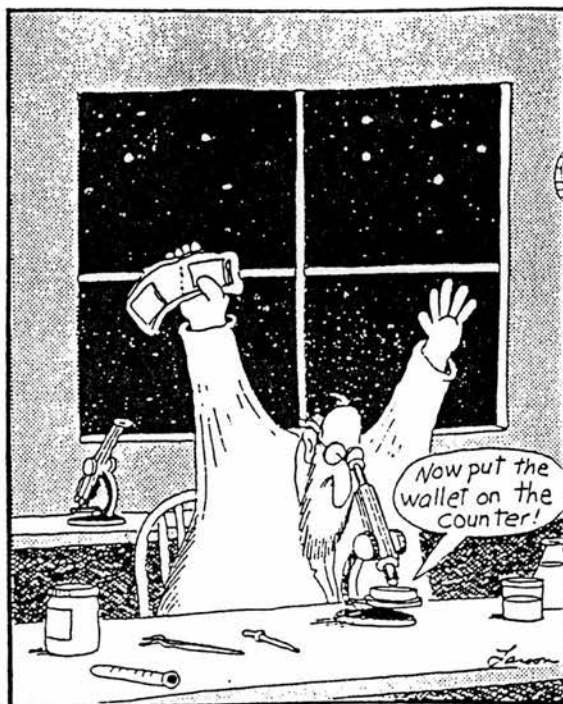
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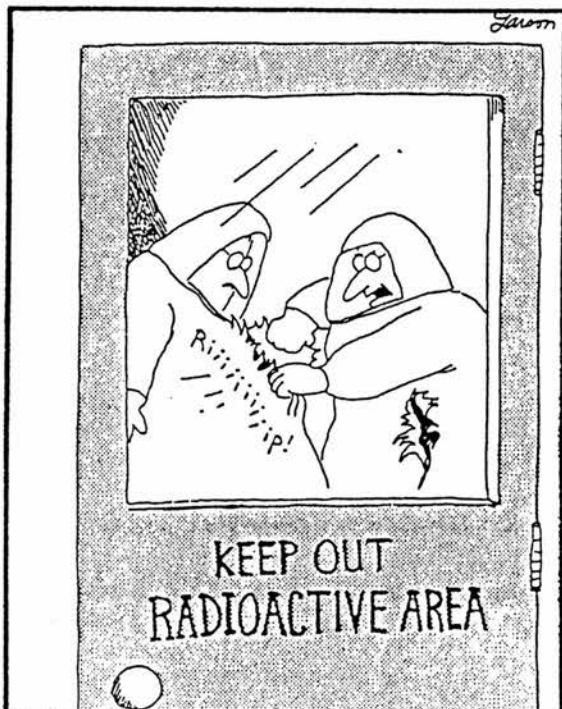
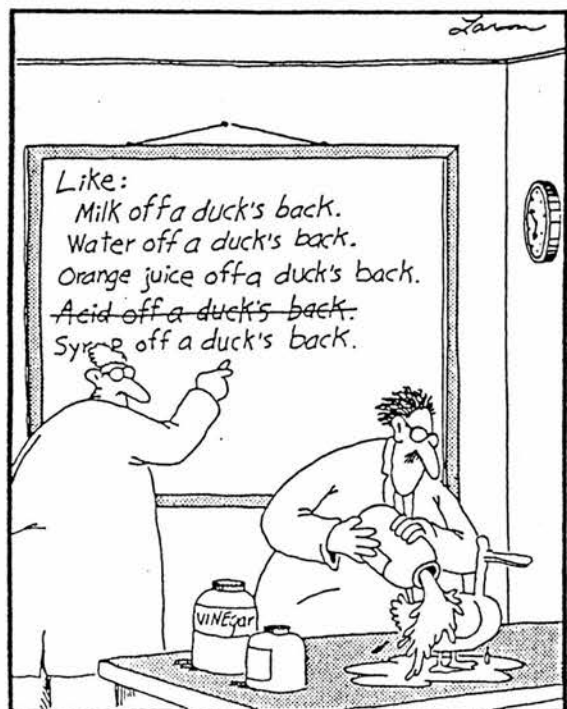
LIFE IN THE LABORATORY



Laboratory peer pressure



Working alone, Professor Dawson stumbles into a bad section of the petri dish.



"So, Foster! That's how you want it, huh? ... Then take THIS!"